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Full length papers will be published approximately four months, and short communications in six to eight weeks, after final acceptance.

In addition to original research papers, the journal will offer immediate publication for Short Communications. Letters to the Editors, offering comment on previous articles, will also be accepted, when the contribution is considered warranted by the Editors.

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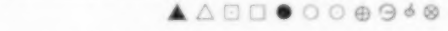

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2. S. T. YOFFE and A. N. NESMEYANOV, *Handbook of Magnesium-Organic Compounds* p. 380. Pergamon Press, London (1956).

Footnotes

Footnotes, as distinct from literature references, should be indicated by the following symbols: *, †, ‡, §, commencing anew on each page; they should not be included in the numbered reference system.

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URINARY EXCRETION OF HYDROXYSTEROIDS*, 17-KETOSTEROIDS AND ALDOSTERONE IN RATS DURING A CYCLE OF TREATMENT WITH MORPHINE

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(*Received* 10 *August* 1960)

Abstract—The effects of morphine and nalorphine on excretion of hydroxysteroids (3 : 21-hydroxysteroids), 17-ketosteroids and aldosterone have been examined in rats. A short cycle of treatment (5–10 days) with morphine (15–75 mg/g body weight daily) has determined an increase of urinary levels of hydroxysteroids. A longer term treatment markedly decreased all steroids in urine as long the injected doses of morphine has shown a "stabilizing" effect. The depressing effect of morphine on adrenal steroid excretion appeared to be only slightly reduced by ACTH injections. Withdrawal of morphine as well as nalorphine injection induced a striking and sustained increase of both aldosterone and hydroxysteroids, whereas 17-ketosteroids attained normal levels. Nalorphine alone reduced the urinary steroids very slightly. A combination of morphine and nalorphine did not produce the same behaviour of urinary steroids previously observed in morphinized rats.

SINCE administration of certain steroids has been found to modify the analgesic effect of morphine and the development of tolerance for the drug,[1,2,3,5] the possibility cannot be rejected that during a repetitive morphine administration a new pattern in production and metabolism of endogenous steroids occurs and may account for the development of a modified behaviour towards the drug.

Previously we have reported[4] that rats briefly treated with a single daily dose of morphine (15 mg/kg body weight) excreted more hydroxysteroids and less 17-ketosteroids and conjugated forms, whereas animals which had long been fully tolerant to the analgesic effect of morphine excreted 17-ketosteroids at approximately normal levels and hydroxysteroids much above control values.

The present paper, which is a comprehensive account of our experiments on this subject from 1958, deals with urinary excretion of steroids in rats to different dosage levels of morphine, to the stage of treatment, and to the withdrawal of the drug or abstinence from nalorphine. For this purpose various rat strains were tested and estimation of steroids in urine was performed by properly sensitive methods. In some experiments the response of adrenal cortex of morphinized rats to injected ACTH or to cold was also investigated.

* According to Weinman and Jayle method in rat urine we found mainly 3 : 21-hydroxysteroids. This agrees with the observation that rat adrenals produce almost entirely corticosterone (Bush; Morris and Williams[12]).

METHODS

Seventy adult rats of the Sprague–Dawley strain and ten male rats of the following strains: Wistar, Long Evans, Swiss albino rats (weighing 150 ± 20 g) were used.

Influence of strain, diet, ambient temperature and relative humidity on basal excretion of steroids in urine was preliminarily examined to select proper experimental conditions (Tables 1 and 2). Fifty-five normal Sprague-Dawley rats, grouped as for experiments with morphine, were kept in separate metabolism cages on standard diet (ambient temperatures 20 ± 2 °C, relative humidity 70 ± 10 per cent) and studied for urinary excretion of steroids during 40 days. Each group of rats was then given morphine.

Two groups of twelve rats received, respectively, 25 mg and 75 mg/kg body weight of morphine daily, for 40 days; one group of seven rats received 10 mg/kg of morphine daily for 140 days; two groups of six rats received, respectively, 15 mg and 30 mg/kg of morphine daily for 40 days; three groups of four rats received, respectively, 5 mg and 20 mg/kg of morphine or 20 mg/kg of morphine plus 20 mg/kg of N-allilnormorphine daily for 40 days.

All rats (excepting 140-day treated animals) were autocontrols. Four normal rats received 50 mg/kg of N-allilnormorphine daily for 40 days. Morphine was injected i.p. as sulphate; N-allilnormorphine was injected i.p. as bromidrate.

Effects of cold and ACTH on adrenocortical steroid excretion were studied, respectively, by abruptly lowering the ambient temperature to 5 °C for 5 hr, and by injecting ACTH intraperitoneally at 15 I.U./kg body weight.

Hydroxysteroids and 17-ketosteroids were analysed on 72-hr collections of urine from each rat; in attempting to estimate urinary aldosterone, the detection of this steroid was performed on reunited fractions from 8-day collections of urine of each rat. Moreover when striking variations of urinary steroids were to be expected, as after ACTH injection, cold and on withdrawal, reunited 24-hr collections of urine from three rats or eight rats were analysed for hydroxysteroids, 17-ketosteroids and aldosterone, respectively, provided in the same manner for controls.

The samples of urine were hydrolysed and extracted for chromatography. A combination of acid and enzymatic hydrolysis was performed according to Romanoff *et al.*[6] After hydrolysis, 0·5 mg of purified NaCl was added to urinary specimens and extraction was made using a mixture of benzene–chloroform–ether (1:1:1) to avoid troublesome emulsions according.[7] The extracts were taken up in 50% ethanol, and passed through a kieselguhr reversed-phase partitional column pretreated with vapour phase of dialkyldichlorosilane, as described by Howard and Martin,[8] using benzene–ether for the stationary phase and 50% ethanol for the mobile phase. This column extracted very efficiently the interfering pigments.

Column chromatography

The first 6 ml of column effluent were collected, evaporated to dryness *in vacuo*, dissolved in 0·1 ml of equilibrated 25% ethanol and transferred to the analytical column for partitional chromatography. The column chromatography was carried out with a kieselguhr column packed according to Butt *et al.*[9] using 25% equilibrated ethanol as stationary phase and toluene as mobile phase. Only the most polar steroids were fractionated by this column,* whereas the less polar steroids (i.e. 17-ketosteroids),

* Aldosterone and hydroxysteroids.

TABLE 1. URINARY STEROIDS IN RATS RESPECTING TO SOME BASAL CONDITIONS

Basal conditions	Urinary steroids (μg/24 hr $\pm$ s.d.)					
	Hydroxysteroids		17-Ketosteroids		Aldosterone	
	mean	range	mean	range	mean	range
(a) Strain*						
Sprague–Dawley	29·3 ± 2·5	(23·5 – 34·5)	6·9 ± 1·0	(5·5 – 8·3)	0·23 ± 0·02	(0·15 – 0·30)
Wistar	36·5 ± 2·4	(32·5 – 40·8)	6·2 ± 1·8	(3·7 – 9·8)	0·28 ± 0·06	(0·20 – 0·35)
Long Evans	51·7 ± 2·0	(47·5 – 56·3)	2·4 ± 1·1	(0·8 – 3·9)†	0·28 ± 0·07	(0·25 – 0·38)
Commercial albino rats	15·2 ± 4·0	(10·3 – 22·2)	14·4 ± 1·7	(8·8 – 15·7(25))‡	0·12 ± 0·04	(0·10 – 0·20)
(b) Diet (Sprague–Dawley)						
Diet *ad libitum*	31·1 ± 2·0	(28·7 – 34·5)	7·3 ± 1·2	(5·5 – 10·4)		
Standard diet	29·3 ± 2·5	(23·5 – 34·5)	6·9 ± 1·0	(5·5 – 8·3)	0·23 ± 0·02	(0·15 – 0·30)
(c) Ambient temperature (Sprague–Dawley)						
20 ± 2 °C	29·3 ± 2·5	(23·5 – 34·5)	6·9 ± 1·0	(5·5 – 8·3)	0·23 ± 0·02	(0·15 – 0·30)
25 ± 2 °C	24·2 ± 2·2	(22·5 – 26·4)	5·6 ± 0·8	(7·5 – 3·4)		
(d) Relative humidity (Sprague–Dawley)						
45 per cent	31·0 ± 2·0	(28·7 – 32·7)	7·2 ± 0·7	(6·5 – 10·3)		
70 per cent	29·3 ± 2·5	(23·5 – 35·5)	6·9 ± 1·0	(5·5 – 8·3)	0·23 ± 0·02	(0·15 – 0·30)

* At 20 ± 2 °C, standard diet and relative humidity 70 per cent (ten rats for each strain).
† Low conjugation activity of steroids was found in Long Evans strain.
‡ Some rats excrete an unidentified 11–17-dioxysteroid.

running practically with toluene front, were collected in the first 3–4 ml of column effluent. Aldosterone and hydroxysteroids were separated and collected respectively in the fifth to eighth and tenth to fifteenth millilitre of column effluent.

Estimation of steroids in single fractions

An aliquot of each fraction was analysed by the following methods: (i) Weinmann and Jayle[10] method (a modification of the original Hill method) for hydroxysteroids (sensibility = 15 μg $\pm$ 3 per cent); (ii) Masson and Corcoran method[11] for α-ketolic steroids; (iii) polarography of Girard hydrazones for Δ^4-3-keto steroids and 17-ketosteroids, according to Morris and Williams[12]. The last two methods have been used by the authors to detect steroids in rat plasma or urine.

Paper chromatography

An aliquot of the various collected fractions from the chromatograph column were put on Whatman no. 4 filter paper for chromatography, cut according to Rubin *et al.*[13]

TABLE 2. COMPOSITION OF THE DIET

Main constituents (g/kg diet)		Vitamins (mg/kg diet)		Vitamins (mg/kg diet)		Vitamins (mg/kg diet)	
Vitamin free		Vit. A,	50	Pantothenic ac,	100	Vit. B_{12},	0·02
Casein,	220	Vit. D,	20	Niacinamide,	200	Vit. C,	1000·00
Starch,	630	Vit. E,	500	Choline,	2000		
Vegetable,		Vit. K,	10	Inositol,	1000		
Oils,	100	Vit. B_1,	50	PABA,	300		
Salt mixture,	10	Vit. B_2,	20	Biotin,	1		

Descending chromatograms of the most polar steroids (i.e. hydroxysteroids and aldosterone) were run with toluene, as mobile phase, previously equilibrated with 75% methanol. The 17-ketosteroids ones were run by the ligroin-propylene glycol equilibrated system at a temperature maintained at 23 ° $\pm$ 2 °C, according to Savard.[14] Parallel chromatograms of authentic samples of each steroid were run in the same manner.

Spot detection in chromatograms

In each chromatogram steroid spots were detected by means of the following reactions: (i) tetrazolium method for α-ketol grouping in C_{17} (using nitro bleu tetrazolium, NBT, in place of bleu tetrazolium to make it suitable for quantities down about 0·1 μg/cm²) according to Chen and Twell;[15] (ii) Zimmerman reaction for 17-ketosteroids according to Savard[16] (sensibility = 2·5 μg/cm²); (iii) Pincus method (antimony trichloride) for hydroxysteroids (sensibility 2·5 μg/cm²);[18] (iv) NaOH fluorescence according to Simpson and Tait[17] for Δ^4-3-ketosteroids (0·25 –1 μg/cm² sensibility).

Alternatively, undeveloped spots were eluted and analysed for ketosteroids by polarography of Girard's hydrazones and by infra-red spectroscopy.

Estimation of aldosterone

Particular attention has been given to the intriguing problem of aldosterone detection in rat urine. Parallel chromatograms of aldosterone column fraction and of

authentic sample of aldosterone were run in the same manner and aldosterone zones were determined by comparison of standard strip on which the spots were developed using the NBT method. Undeveloped spots were eluted with methanol and after solvent evaporation the residue was partitioned between 10 ml of dichloromethane and 2 ml of water, and the water phase discarded.

A first sample (half) of dichloromethane phase was analysed by polarography of Girard hydrazones (sensibility = 0·2 μg)[12].

A second sample was put on chromatographic paper, acetylated and oxidized, and run into two different systems according to Mattox *et al.*[19] Parallel chromatograms of authentic sample of acetylated and oxidized aldosterone were run in the same manner for accurate detection of spots. Alternatively for the purpose of an exclusively qualitative identification, infra-red spectrum of aldosterone eluted spots was recorded and compared with an infra-red curve of standard aldosterone.

Apparatus, and chemicals

For polarographic procedure Sergent's polarograph was used with the micropolarographic cell described by Morris and Williams.[12] For fluorescence analysis Beckman's DU spectrophotometer was employed. Infra-red spectra of steroids were registered in Beckman's infra-red spectrophotometer.

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All the chemicals used, with the exception of standard steroids, were products of Merck, Darmstadt. Highly purified standard steroids were a gift from N. V. Organon, which we gratefully acknowledge.

NaCl for polarographic procedure was recrystalized three times from ethanol to free it from interfering impurities giving a polarographic wave approximately at −1·1 V *tert*-Butanol, used as solvent for the Girard reagent in the polarographic procedure, was purified by ten successive extractions with half-saturated KCl solution, dried and distillated according to Morris and Williams.[12]

A glass development chamber was used for descending development of chromatograms, previously saturated with the vapours of the mobile phase and maintained at a controlled temperature. Paper was saturated with the stationary phase and brought to equilibrium with the atmosphere in the chamber for 24 hr prior to irrigation with solvent. All the chromatograms were run in the machine direction of the paper.

RESULTS

(a) *Urinary steroids in normal rats with respect to some basal conditions*

As shown in Table 1 excretion of steroids was found to vary according to strain, diet, ambient temperature and relative humidity. In the present work an inbred strain, Sprague–Dawley, appeared to be preferable for its homogeneity. On standard diet, atmospheric pressure 760 ± 10 mm Hg, ambient temperature 20 ± 2 °C, relative humidity 70 ± 10%, the urinary levels of steroids were found in this strain to range, for hydroxysteroids from 23·5–34·5 μg/24 hr, for 17-ketosteroids from 5·5 to 8·3 μg/24 hr, and for aldosterone from 0·15 to 0·30 μg/24 hr.

Paper chromatography of hydroxysteroids in toluene–75%methanol has given three spots (X_1, X_2, X_3) on the strip of which the middle one (X_2) was found to run at the same rate of pregnane-3α:11β:21-triol-20-one (Fig. 1). The other spots are still unknown (X_1 and X_3).

Two of the three spots (X_1 and X_2) developed a blue colour with the NBT method and all of them a brown colour with the $SbCl_3$ method showing, respectively, a α-ketolic chain in C_{17} and 3-hydroxy grouping. No reaction occurred with the Zimmerman method nor with NaOH fluorescence, indicating that 17-keto and Δ^4-3 keto groupings are absent. Polarography of Girard hydrazones of eluted X_2 spot have shown the typical polarographic wave of the 20-keto grouping, in agreement with the NBT method, but no 3-keto wave. The infra-red spectrum of this spot has given a curve identical to that recorded from an authentic sample of pregnane-3α:11β:21-triol-20-one. Polarography of eluted X_3 spot has given no polarographic wave showing no keto grouping. This finding was confirmed by infra-red spectrum of eluted spot. The method of Weinmann and Jayle[10] for hydroxysteroids has given 1 μEquiv. of acetylable hydroxy radicals per 180 μg of steroid showing that two acetylable OH radicals probably occur in steroid molecules.

From these findings it may be assumed that the probable structure of this steroid is that of pregnane-3α:20-diol with or without a non-reactive βOH radical in C_{11}.

The X_1 spot has given such a small amount of substance that no investigation was performed on its steroid content.

A two-step paper chromatography of 17-ketosteroids was performed. The paper strips were first chromatographed for 96 hr (Fig. 2). The portion of steroid mixture which has run off the lower end of the chromatograms was rechromatographed on fresh sheets of paper for 24 hr (Fig. 3). In 96-hr chromatograms, two spots were detected (X_4–X_5) with *Rt* closely similar to etiocholanone (X_4) and androsterone (X_5). These spots have given only the Zimmerman reaction. In 24-hr chromatograms another two spots were found by the Zimmerman method (X_6–X_7). The steroids apparently run with the same rate of etiocholan-3α:11β-diol-20-one (X_6) and androstene 3α:11β-diol-20-one (X_7).

(b) *Urinary excretion of steroids in rats chronically treated with morphine at different dosage levels*

According to our findings the schematization of a cycle of treatment with morphine in rats respecting to urinary pattern of steroids and animal behaviour may be suggested. For this purpose we have divided the entire cycle of treatment into three stages: (i) first stage from 0 to 10 days; (ii) second stage from 10 to 40 days; (iii) third stage from 40 to 140 (or more) days. If the dose of morphine is not increased during the last stage of treatment, the third stage apparently resembles the abstinence stage since the injected dose of morphine is no more stabilizing.

First stage (Tables 3–7; Figs. 4–7). At dosage levels of morphine ranging from 15 mg to 75 mg/kg body weight daily, an increase of hydroxysteroids and a decrease of 17-ketosteroids and possibly of aldosterone were observed, whereas at lower dose levels of morphine a slight decrease of the aforementioned steroids has taken place after the onset of treatment. During this stage morphine has induced a full analgesic effect. Moreover the higher injected doses have produced typical "excitatory" phenomena in rats.

Second stage (Tables 3–7; Figs. 4–7). In this stage all the doses injected decreased the urinary excretion of the investigated steroids. At the end of this stage analgesic response to morphine appeared to be lessened although the reaction time to painful stimulus amounted to from one and a half times to twice the normal reaction time.

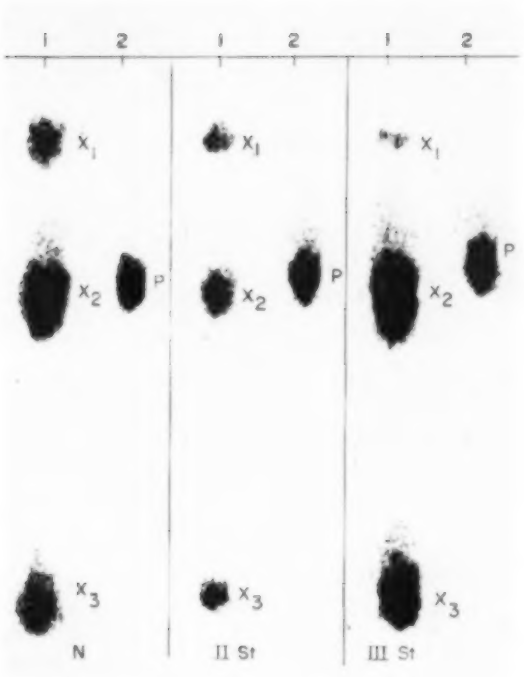

FIG. 1. Fractionation of urinary hydroxysteroids from: N, normal rats; II St, rats under 30 days treatment with morphine; III St, rats under 140 days treatment with morphine. X_1, X_2, X_3, spots of urinary hydroxysteroids; P, spot of standard steroid (pregnane-3α:11-β:21-triol-20-one).

facing page 6

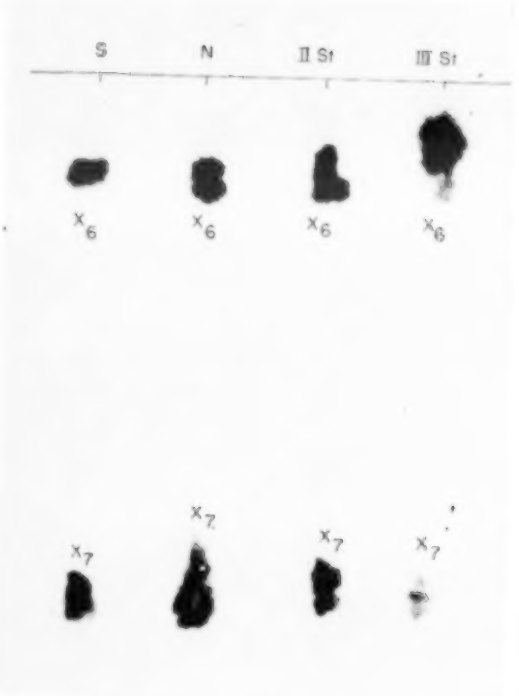

FIG. 3. Fractionation of urinary 17-ketosteroids in 24-hr chromatogram from: N, normal rats; II St, rats under 30 days treatment with morphine; III St rats under 140 days treatment with morphine S, standard steroids; E, ethiocolan-3α : 11β-diol-20-one; A, androstene-3α : 11β-diol-20-one. X_6, X_7, urinary ketosteroids.

FIG. 2. Fractionation of urinary 17-ketosteroids in 96-hr chromatogram from: N; normal rats; II St. rats under 30 days treatment with morphine; III St, rats under 140 days treatment with morphine S, standard steroids, i.e. E, etiocholanone, A, androsterone. X_4, X_5, urinary 17-ketosteroids.

The behaviour of the animals was characterized by reduced activity, loss of weight, ipotermia and unusual incidence of abscesses in skin, lung and peritoneum. Urinary steroid fractionation on the thirtieth day of the treatment by paper chromatography has given a steroid pattern similar to the controls but with lower colour intensity of the developed spots (Figs. 1–3).

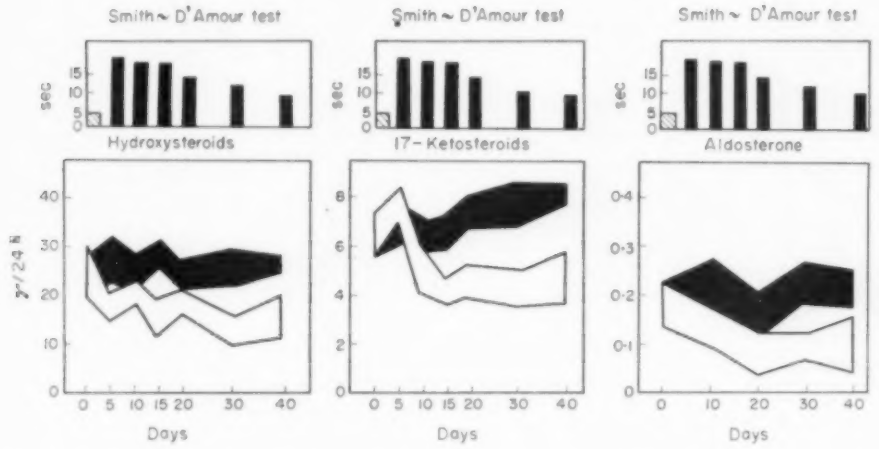

FIG. 4. Range of urinary steroids in rats during a treatment with morphine (5 mg/kg body weight daily). Solid area; controls. Open area; rats under morphine.

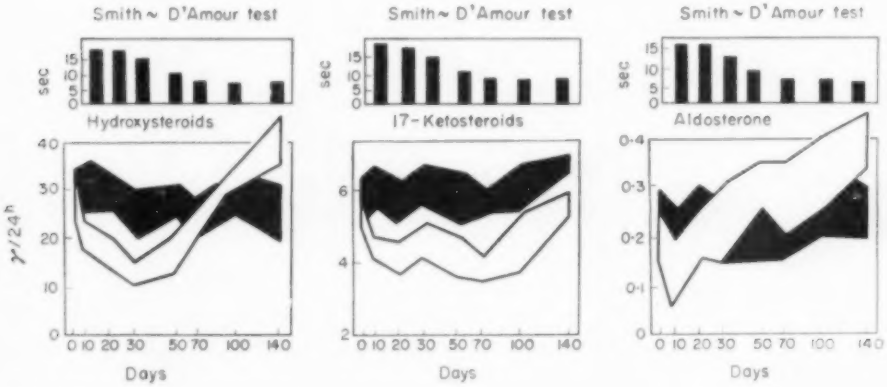

FIG. 5. Range of urinary steroids in rats during a long term treatment with morphine (10 mg/kg body weight daily). Solid area: controls. Open area: rats under morphine.

Third stage (Table 3; Fig. 5). Repeated administration of morphine at the same dose levels (no more stabilizing) induced in rats an aggressive behaviour and eventually cannibalism, while the reaction time to painful stimulus appeared to approximate more and more to the normal values. At this stage the 17-ketosteroids have increased to

TABLE 3. URINARY STEROIDS IN RATS DURING A TREATMENT WITH MORPHINE (10 MG/KG BODY WEIGHT DAILY)

Hydroxysteroids (μg/24 hr ± s.d.)					17 Ketosteroids (μg/24 hr ± s.d.)				Aldosterone (μg/24 hr ± s.d.)				
			P (t test)				P (t test)					P (t-test)	
Days	Controls	Treated	Respecting controls	Between days	Controls	Treated	Respecting controls	Between days	Days	Controls	Treated	Respecting controls	Between days
0	29·2 ± 3·61	29·1 ± 3·50		(0–I) >0·05	5·7 ± 0·40	5·6 ± 0·56		(0–I)	0	0·27 ± 0·02	0·26 ± 0·07		
8–10 (I)	29·5 ± 3·16	24·5 ± 5·68	>0·05	(I–II) <0·05	5·7 ± 0·42	4·4 ± 0·46	<0·001	<0·01 (I–II)	3–10 (I)	0·24 ± 0·03	0·15 ± 0·05	<0·01	(O–I) <0·01
18–20 (II)	29·7 ± 3·10	17·8 ± 4·38	<0·001	(II–III) <0·05	5·6 ± 0·49	3·9 ± 0·81	<0·001	<0·01 (II–III)					(I–III) <0·01
28–30 (III)	26·1 ± 3·16	12·4 ± 6·15	<0·001	(III–IV) <0·001	5·9 ± 0·57	3·3 ± 0·54	<0·001	<0·001 (III–IV)					
68–70 (IV)	26·3 ± 2·90	24·9 ± 1·41	>0·05	(IV–V) <0·001	5·7 ± 0·31	3·8 ± 0·41	<0·001	>0·05 (IV–V)	63–70 (II)	0·25 ± 0·33	0·27 ± 0·05	>0·05	
98–100 (V)	27·5 ± 2·66	32·4 ± 3·16	<0·01	V–VI <0·05	6·0 ± 0·65	4·5 ± 0·47	<0·001	<0·01 (I–VI)					
138–140 (VI)	26·1 ± 3·16	39·8 ± 7·50	<0·001		6·1 ± 0·74	5·2 ± 0·67	<0·05	>0·05	133–140 (III)	0·25 ± 0·09	0·36 ± 0·13	<0·05	

TABLE 4. URINARY EXCRETION OF STEROIDS IN MORPHINE TREATED RATS (15 MG/KG BODY WEIGHT DAILY)

Hydroxysteroids (μg/24 hr ± s.d.)					17 Ketosteroids (μg/24 hr ± s.d.)				Aldosterone (μg/24 hr ± s.d.)				
			P (t-test)				P (t-test)					P (t-test)	
Days	Controls	Treated	Respecting controls	Between days	Controls	Treated	Respecting controls	Between days	Days	Controls	Treated	Respecting controls	Between days
0	27·30 ± 5·29	25·33 ± 4·57	>0·05	(0–I)	6·06 ± 2·28	6·11 ± 0·77	> 0·05	(0–III)	0	0·19 ± 0·05	0·19 ± 0·03		(0–I)
3–5(I)	25·83 ± 2·56	33·66 ± 1·26	<0·001	<0·001	6·11 ± 0·61	5·06 ± 0·47	< 0·01	< 0·001	3–10(I)	0·22 ± 0·04	0·13 ± 0·10	> 0·05	> 0·05
18–20(II)	28·83 ± 2·40	22·83 ± 1·84	<0·001		6·25 ± 0·41	4·53 ± 0·66	<0·001						
38–40(III)	29·16 ± 2·04	19·33 ± 2·44	<0·001	(0–III) <0·05	6·50 ± 0·55	4·01 ± 0·55	<0·001		33–40(II)	0·22 ± 0·03	0·10 ± 0·03	<0·01	(0–II) <0·01

reach about the normal level whereas the hydroxysteroids and the aldosterone increased above the starting levels.

Paper chromatograms of steroids on the 140th day of treatment have given a modified pattern. Chromatograms of hydroxysteroids have shown a paler X_1 spot and an increase in colour and size of the X_2 and X_3 spots. Ultra violet analysis of NaOH-sprayed chromatograms revealed a new fluorescent spot of small size near the lower

TABLE 5. URINARY HYDROXYSTEROIDS AND 17-KETOSTEROIDS IN MORPHINE TREATED RATS (20 MG/KG BODY WEIGHT DAILY)

	Hydroxysteroids (μg 24/hr)*		17-Ketosteroids (μg/24 hr)*	
Days	Normal	Treated	Normal	Treated
0	31·5 ± 4·53	29·25 ± 5·56	6·37 ± 0·94	6·57 ± 0·43
3–5	27·5 ± 1·26	35·73 ± 2·88	6·62 ± 0·85	4·37 ± 0·74
18–20	31·5 ± 4·63	21·75 ± 5·56	7·00 ± 0·91	3·85 ± 0·62
38–40	30·2 ± 4·19	20·00 ± 4·07	7·12 ± 0·94	3·55 ± 0·40

* ± s.d.

end of chromatograms. The NaOH fluorescence of the spot reveals 3-keto grouping but its steroid structure remains unknown. Chromatograms of 17-ketosteroids have shown X_4–X_5 spots similar to those of the second stage. Moreover the size and colour intensity of X_6 have increased while those of the X_7 have decreased, the size and colour intensity of X_6 having increased (Figs. 1–3).

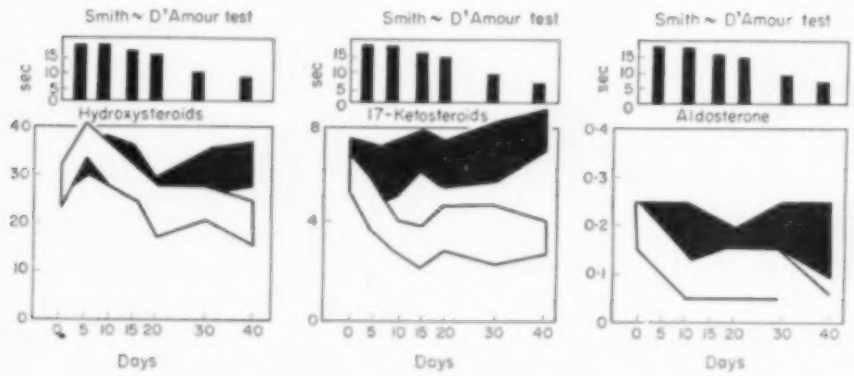

FIG. 6. Range of urinary steroids in rats during a treatment with morphine (20 mg/kg body weight daily). Solid area: controls. Open area: rats under morphine.

(c) *Urinary excretion of steroids in rats during abstinence syndrome* (*Tables* 8 *and* 9)

In the second stage of treatment the withdrawal of stabilizing doses of morphine so as nalorphine injection have induced a tremendous increase of hydroxysteroids and possibly of aldosterone in rat urine, whereas the 17-ketosteroids have attained normal levels.

TABLE 6. URINARY STEROIDS IN RATS DURING A TREATMENT WITH MORPHINE (25 MG/KG BODY WEIGHT DAILY)

Hydroxysteroids (μg/24 hr ± s.d.)*					17-Ketosteroids (μg/24 hr ± s.d.)*				Aldosterone (μg/24 hr ± s.d.)†				
			P (t test)				P (t-test)					P (t-test)	
Days	Controls	Treated	Respecting control	Between days	Controls	Treated	Respecting controls	Between days	days	Controls	Treated	Respecting controls	Between days
0	28·5 ±6·63	27·2±3·5		(0–I) < 0·001	6·40±0·34	6·8±1·1		(0–I) < 0·001	0	0·24±0·03	0·23±0·02		(0–I) > 0·05
8–10(I)	26·6 ±4·48	35·4±2·6	< 0·01	(I–II) < 0·001	7·07±0·44	5·2±0·4	< 0·001	(I–II) < 0·001	3–10(I)	0·22±0·22	0·16±0·17	> 0·05	
18–20(II)	29·3 ±3·90	26·3±3·7	> 0·05		7·01±0·51	3·9±0·5	< 0·001						
38–40(III)	28·5 ±3·71	14·4±1·8	< 0·001	(II–II) < 0·001	7·11±0·90	4·3±0·3	< 0·001	(II–III) > 0·05	33–40(II)	0·25±0·03	0·10±0·03	< 0·001	(0–II) < 0·001

* Ten autocontrol rats.
† Eight autocontrol rats.

TABLE 7. URINARY STEROIDS IN RATS DURING A TREATMENT WITH MORPHINE (75 MG/KG BODY WEIGHT DAILY)

Hydroxysteroids (μg/24 hr ± s.d.)*					17-Ketosteroids (μg/24 hr ± s.d.)*				Aldosterone (μg/24 hr ± s.d.)†				
			P (t-test)				P (t-test)					P (t-test)	
Days	Controls	Treated	Respecting controls	Between days	Controls	Treated	Respecting controls	Between days	Days	Controls	Treated	Respecting controls	Between days
0	27±2·2	28·1 ±3·50		(0–I) < 0·001	6·71±1·5	6·85±1·1		(0–I) > 0·05	0	0·21±0·03	0·22±0·02		(0–I) > 0·05
3–5(I)	26±1·8	38·6 ±2·80	< 0·001	(I–II) < 0·001	7·32±1·1	7·10±0·3	> 0·05	(I–II) < 0·01	3–10(I)	0·22±0·24	0·16±0·09	> 0·05	
18–20(II)	26±1·6	15·4 ±1·79	< 0·001	(II–III) < 0·001	6·88±1·7	4·30±0·7	< 0·001	(II–III)					
38–40(III)	27±2·0	10·9 ±3·60	< 0·001		7·17±1·4	3·90±0·6	< 0·05		33–40(II)	0·22±0·24	~0·03‡		

* Ten autocontrol rats.
† Eight autocontrol rats.
‡ Measures about the method sensibility limit.

On withdrawal, or soon after nalorphine, rat abstinence syndrome[20] has developed rapidly, animals were depressed, voided and frequently excreted semiliquid evacuations. During withdrawal, tolerance to the analgesic effect of morphine was slowly lost over a period of about 10 days, whereas after nalorphine injection tolerance disappeared rapidly (a few days). After withdrawal, urinary levels of hydroxysteroids and

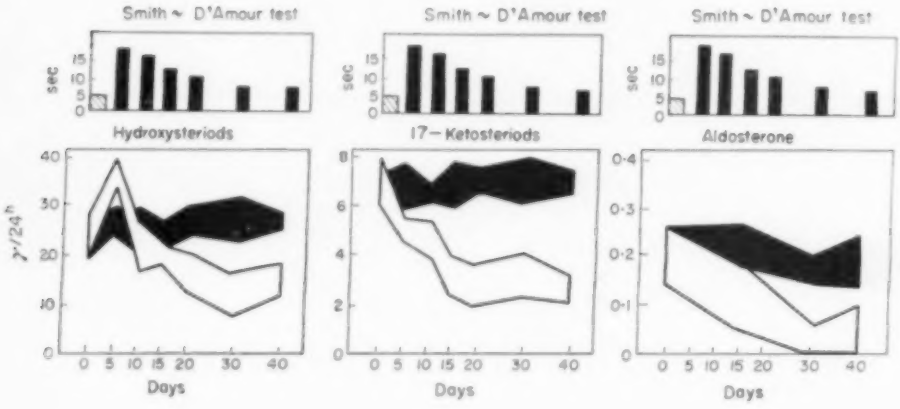

FIG. 7. Range of urinary steroids in rats during a treatment with morphine (30 mg/kg body weight daily). Solid area: controls. Open area: rats under morphine.

TABLE 8. URINARY EXCRETION OF STEROIDS IN RATS ON WITHDRAWAL OF MORPHINE*

Morphine	Days of treatment	Days of withdrawal	No. of samples†	Hydroxysteroids (μg/hr)‡	17-Ketosteroids (μh/24 hr)	No. of samples‡	Aldosterone (μg/24 hr)
—	—	—	3	29·2 ± 3·6	6·4 ± 0·3	1	0·25
30	40	—	2	15·3 ± 2·8	2·8 ± 0·8	—	—
30	40	3	2	80·3 ± 13·2	12·6 ± 2·8	—	—
30	40	5	2	65·4 ± 9·6	8·5 ± 1·8	—	—
30	40	8	2	55·6 ± 7·4	7·4 ± 1·7	—	—
30	40	13	2	41·3 ± 6·5	6·7 ± 1·8	—	—
75	40	—	3	13·2 ± 3·0	2·2 ± 0·9	1	0·1
75	40	3	3	102·5 ± 22·4	8·3 ± 2·2	1	0·5
75	40	5	3	90·3 ± 11·2	10·2 ± 2·6	1	0·4
75	40	8	3	70·5 ± 9·6	7·6 ± 1·8	1	0·3
75	40	13	3	55·7 ± 7·5	6·9 ± 1·8	1	0·3

* average daily excretion ± s.d.
† One sample = reunited 24-hr samples of urine from three rats.
‡ One sample = reunited 24-hr samples of urine from eight rats.

aldosterone remained high and attained normal values on approximately the thirteenth to fifteenth day, whereas after nalorphine injection complete steroid recovery was accomplished on about the fifth day.

TABLE 9. URINARY EXCRETION OF STEROIDS IN MORPHINIZED RATS ON ABSTINENCE INDUCED BY NALORPHINE

Dose of morphine mg/kg per hr	Days of treatment	Dose of nalorphine (mg/kg)	Days of abstinence	No. of samples*	Hydroxy-steroids (μg/24 hr $\pm$ s.d.)	17-Keto-steroids (μg/24 hr $\pm$ s.d.)	No. of samples†	Aldosterone (μg/24 hr $\pm$ s.d.)
—	—	—	—	4	28·5 ± 6·6	6·2 ± 0·4	1	0·3
25	40	—	—	4	10·4 ± 1·8	4·3 ± 0·3	1	0·1
25	40	50	1	4	105·5 ± 24·2	10·5 ± 3·5	1	0·7
25	40	50	3	4	38·2 ± 12·9	8·7 ± 1·6	1	0·3
25	40	50	5	4	25·3 ± 8·7	9·2 ± 1·8	1	0·2
75	40	—	—	1	12·2	4·1	—	—
75	40	50	1	1	85·5	10·4	—	—
75	40	50	3	1	45·3	7·5	—	—
75	40	50	5	1	31·5	5·3	—	—

* One sample = reunited 24 hr samples of urine from three rats.
† One sample = reunited 24 hr samples of urine from eight rats.

(d) *Effects of ACTH and cold on urinary excretion of adrenocortical steroids in morphinized rats*

Table 10 records urinary levels of hydroxysteroids aldosterone and 17-ketosteroids from morphinized rats exposed to low ambient temperature (5 °C for 5 hr).

In rats acting as untreated controls, the cold markedly increased the urinary excretion of hydroxysteroids and slightly that of the 17-ketosteroids; levels of aldosterone appear to be unaffected.

TABLE 10. EFFECT OF COLD ON URINARY STEROIDS IN RATS CHRONICALLY TREATED WITH MORPHINE (25 DAYS)

Morphine	Temperature (°C)	No. of samples*	Hydroxysteroids (μg/24 hr ± s.d.)	17-Ketosteroids (μg/24 hr ± s.d.)	No. of samples†	Aldosterone (μg/24 hr ± s.d.
	20	4	28·70 ± 3·22	5·71 ± 0·48	1	0·20
	5	4	82·80 ± 7·25	9·12 ± 0·76	1	0·22
15	20	2	15·02 ± 2·10	3·15 ± 0·78	—	—
	5	4	47·85 ± 3·75	5·35 ± 1·62	—	—
25	20	4	17·01 ± 3·21	2·82 ± 0·74	1	0·15
	5	4	28·92 ± 2·45	3·42 ± 0·74	1	0·15

* One sample = reunited 24-hr samples of urine from three rats.
† One sample = reunited 24-hr samples of urine from eight rats.

Morphine did not inhibit the effects of cold on steroid excretion, but the increase of urinary levels of hydroxysteroids and 17-ketosteroids achieved in rats to which morphine was given and which were exposed to cold, did not attain the high values obtained from the untreated controls (Table 11).

TABLE 11. INCREASE OF URINARY EXCRETION OF HYDROXYSTEROIDS + 17-KETOSTEROIDS IN NORMAL AND MORPHINIZED RATS (25 MG/KG BODY WEIGHT) AFTER TEMPERATURE LOWERING

Samples*	Treatment	Increase†	*P* (*t*-test)
4	—	57·0 ± 6·16	<0·001
4	Morphine	12·2 ± 4·39	

* Reunited 24-hr samples of urine from three rats.
† μg/24 hr ± s.d.

Table 12 depicts the rise of urinary hydroxysteroids and 17-ketosteroids we have found in normal rats injected with ACTH. In morphinized rats the same dose of ACTH has also increased the urinary hydroxysteroids and the 17-ketosteroids although at very lower levels than in normal animals (Table 13). Results suggested that the low levels of urinary corticosteroids achieved in morphinized rats exposed to cold may be more strictly related with the lessened response of adrenals to ACTH than with a decrease in hormone released from hypophysis.

Table 12. Effect of ACTH on urinary excretion of steroids in rats chronically treated with morphine (28 days)

Morphine (mg/kg)	ACTH (15 U/kg i.p.)	No. of samples*	Hydroxy-steroids (μg/24 hr ± s.d.)	17-Keto-steroids (μg/24 hr ± s.d.)	No. of samples†	Aldosterone (μg/24 hr ± s.d.)
—	—	4	29·60 ± 3·10	5·80 ± 0·62	1	0·25
—	0·1	4	176·17 ± 25·85	22·72 ± 6·16	1	0·28
15	—	2	14·85 ± 3·04	3·10 ± 0·84	—	—
15	0·1	4	88·90 ± 8·91	9·60 ± 1·55	—	—
25	—	4	15·15 ± 2·18	3·25 ± 0·74	1	0·15
25	0·1	4	50·52 ± 4·71	11·97 ± 5·40	1	0·15

* One sample = reunited 24-hr samples of urines from three rats.
† One sample = reunited 24-hr samples of urine from eight rats.

Table 13. Increase of urinary excretion of hydroxysteroids + 17-ketosteroids in normal and morphinized rats (25 mg/kg body weight) after ACTH

Samples*	Treatment	Increase†	*P* (*t*-test)
4	—	163·44 ± 58·55	<0·01
4	Morphine	44·15 ± 4·81	

* Reunited 24 hr samples of urine from three rats.
† μg/24 hr ± s.d.

Table 14. Urinary hydrosteroids and 17-ketosteroids in morphine plus nalorphine treated rats (20 mg/kg body weight respectively daily)

Days	Hydroxysteroids (μg/24 hr ± s.d.)		17-Ketosteroids (μg/24 hr ± s.d.	
	Normal	Treated	Normal	Treated
0	31·20 ± 2·51	30·25 ± 2·88	6·30 ± 0·48	6·37 ± 2·00
3–5	30·10 ± 3·00	26·25 ± 2·88	5·30 ± 0·50	5·25 ± 0·60
18–20	27·25 ± 2·07	26·25 ± 1·73	6·45 ± 3·31	5·25 ± 0·45
38–40	30·25 ± 3·31	24·50 ± 3·09	6·57 ± 2·75	4·02 ± 0·68

(e) *Urinary excretion of steroids in rats given nalorphine alone or morphine plus nalorphine*

Only a slight decrease of steroids appeared after a long term treatment with nalorphine alone (Fig. 8). Rats given morphine (20 mg/kg) plus nalorphine (20 mg/kg) (Table 14, Fig. 9) excreted hydroxysteroids, 17-ketosteroids and aldosterone at higher levels than animals under morphine alone (Table 5, Fig. 6). Moreover the increase of hydroxysteroids previously observed in the first stage of treatment with morphine, at the same dose, was no longer noted after combined administration of morphine and nalorphine. During combined treatment the analgesic effect of morphine appeared to be lessened.

DISCUSSION

Although many researchers have attentively studied the complex problem of the influence of morphine on adrenal and gonad functions, few and conflicting data have been reported on the subject of production, metabolism and excretion of hormonal steroids under morphine.

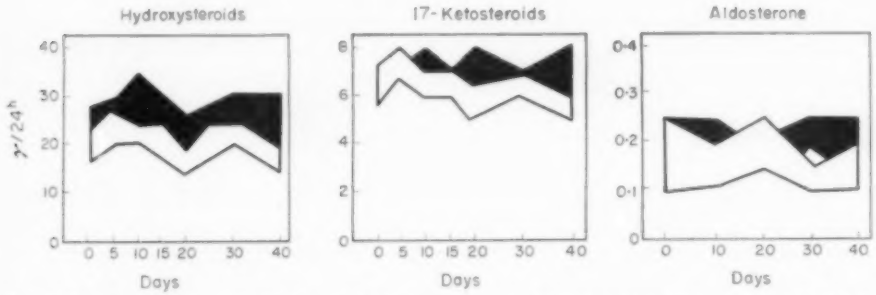

FIG. 8. Range of urinary steroids in rats during a treatment with nalorphine (50 mg/kg body weight). Solid area: controls. Open area: rats under nalorphine.

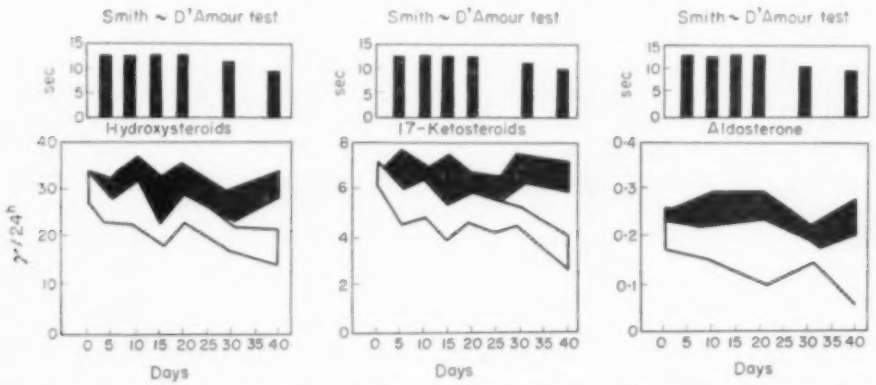

FIG. 9. Range of urinary steroids in rats under a combined treatment with morphine plus nalorphine (20 mg/kg and 20 mg/kg body weight, respectively). Solid area: controls. Opens area: rats under combined treatment.

Single doses of morphine increase in the cat the excretion of 17-ketosteroids.[21] In post-addict men[22] single subcutaneous injections of morphine as large as 45–80 mg induced a decrease of urinary 17-ketosteroids ranging from 35 to 40 per cent; higher doses, however, achieved lesser depressant effects and sometimes "erratic responses" on 17-ketosteroid excretion, indicating an adrenocortical stimulation indeed of a depression. No data are reported on the effects of analgesic doses of morphine on steroids.

The reports on the urinary pattern of glyco and mineralcorticoids after a single dose of morphine are scanty. In man an increase as well as a decrease of blood and urine 17-hydroxysteroids has been occasionally observed.[22, 23] No report is available on the production and metabolism of testicular and adrenal steroids under chronic morphine in animals.

In the case of two patients, 17-hydroxycorticosteroids[23] were found to decrease during a cycle of addiction and to increase after withdrawal. In nine volunteers addicted to morphine through increasing doses of the drug,[22] stabilizing doses of 240 mg–360 mg produced an average decrease of 50 per cent in 17-ketosteroid excretion. Withdrawal accomplished an average increase of 17-ketosteroids in the urine. Some subjects, however, showed no increase nor decrease of urinary steroids.

According to our previous work,[4] injection of high doses of morphine in untreated rats may induce an increase of urinary hydroxysteroids. Lower doses produced lower effects on steroids. With regard to the effect of doses these results agree quite well with the observations of George and Way[24] through ascorbic acid fall test in rats. Moreover all the applied doses have reduced without exception the urinary excretion of 17-ketosteroids.

The increase of hydroxysteroids observed in the first stage of treatment may well be the result of a stimulatory effect of morphine on hypophysis eventually stressing in nature, since it is found to be lacking after small doses of the drug.

With reference to the depression of urinary steroids observed in the second stage of treatment by "stabilizing" doses of morphine the opinion of a reduction of urinary adrenal steroids as due exclusively to an impairment of hypophysis by morphine, is hardly sustainable in the rat, since either ACTH injection or the exposure to cold failed to have the same effect on urinary steroids as in untreated animals. This suggests that under morphine the target glands are less responsive to proper corticotropic stimulation. On the contrary the responsibility of ACTH may be invoked to explain the urinary increase of hydroxysteroids observed shortly after withdrawal or nalorphine injection in morphinized rats. The problem of the function of the aforementioned hormonal derangement with reference to the biological disposition to tolerance, addiction and abstinence is quite intricate. Selye[25] reports that adrenalectomy does not impair the ability of rats tolerant to morphine to withstand otherwise lethal doses of the drug. Tanabe and Cafruny[26] observed acquired tolerance to increasing doses of morphine in hypophysectomized rats. The course of withdrawal appeared not to be modified with respect to normal rats.

Moreover, in human addicts Fraser and Isbell[27] have found that a treatment with cortisone or ACTH tends to hasten and aggravate the appearance of withdrawal symptoms. Winter and Flataker[5] have shown an increase of antagonistic effects of nalorphine by cortisone in rats. In the present work the increase of urinary corticosteroids was found to parallel the course of abstinence syndrome.

It may be noted that an increase of urinary steroids also occurs when the injected doses of morphine are not "stabilizing". However, though still obscure as are most of the biological events affected by morphine, the relations between the course of experimental morphinism and hormonal derangements induced by the drug appear worthy of further investigations.

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INHIBITORY EFFECT OF MORPHINE ON METABOLISM OF ADRENAL AND TESTICULAR STEROIDS

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Abstract—Adrenal slices from morphinized rats produce steroid hormones to a minor extent respecting controls, when incubated *in vitro* under proper conditions. After morphine additions of ACTH did not produce such an increase of the hormone production as realized in controls. Morphine when added *in vitro* to adrenal and testicle homogenates from normal rats, appeared to act as an inhibitor of 3-beta-ol-dehydrogenase system. The rate of conjugation of DOC by liver slices excised from morphinized rats was decreased.

INTRODUCTION

IN A previous work[1] we have shown that in morphine-treated rats the urinary excretion of adrenal and testicular hormones displays a characteristic pattern according to the stage of treatment. When "stabilizing" doses were injected for a sufficient period, the amounts of hydroxysteroids, 17-ketosteroids and aldosterone in the urine decreased to a great extent. ACTH administration induced a lesser increase than normal of steroid excretion as target glands were unresponsive to proper hypophyseal stimulation. Moreover under morphine the conjugated forms of steroids appeared to be decreased in urine.

In the present work attention was focused on the possible existence of a direct effect of morphine on steroid metabolism by adrenal and testicular tissue, as well as on the occurrence of an inhibitory effect of the drug on liver corticosteroid conjugation.

In the present work the following situations have been studied:

(1) *In vitro*, hormone production by adrenal slices prepared from glands of morphinized rats.

(2) Influence of morphine on the stimulatory effect of added ACTH on corticosteroid hormone production by adrenal cortical slices.

(3) Effects of morphine on the conversion of Δ^4-3 hydroxysteroids into Δ^4-3 keto compounds by whole homogenates of adrenals and testicles.

(4) The rate of disappearance of Δ^4-3 keto grouping from corticosteroids by liver slices excised from morphinized rats.

Investigation on 3-beta-ol-dehydrogenase system was suggested by the emphasis that the increase of Sudan stained drops in adrenal cortex, as observed after chronic morphine by various authors and by us[2, 3] may depict the failure of the gland to produce hormonal steroids from precursors.

METHODS

Twenty male rats of the Sprague–Dawley strain weighing 150–180 g and maintained in the same ambient conditions as previously described[1] were given morphine intraperitoneally at the dose of 20 mg/kg for 5, 15 and 30 days.

Corticosteroid hormone production by adrenal slices was investigated according to Saffran *et al.*[4] ACTH was added to adrenal slices at a concentration of 1 I.U./g tissue. The enzymic action of 3-beta-ol-dehydrogenase was investigated according to Samuels *et al.*[5] in whole homogenates of adrenals and testicles excised from normal Sprague–Dawley rats. Morphine was added to the medium at concentrations of 50 and 100 μg/g tissue. Liver conjugation of DOC was estimated according to Louchart and Jailer[6] in normal and tolerant rats.

RESULTS

(1) Table 1 shows an inhibitory effect of the previous treatment with morphine on hormone production by adrenal slices under incubation conditions previously described by Saffran *et al.*[4] As may be observed, a short treatment corresponds to a slight

TABLE 1. CORTICOSTEROID PRODUCTION BY ADRENAL SLICES FROM MORPHINIZED RATS, UNDER BASAL CONDITIONS AND AFTER INCUBATION WITH ACTH

Morphine (mg/kg)	Days of treatment	No. of adrenals	$\alpha:\beta$-Unsaturated ketone† Preincubation samples*	(μg/g per 3 hr)	ACTH, 1 I.U./g samples*	(μg/g per 3 hr)
—	—	14	2	73·7 ±1·79	2	211·0 ±17·08
20	5	12	2	80·0 ±2·82	2	201·0 ± 4·94
20	15	14	2	50·5 ±3·53	2	71·0 ± 8·48
20	30	14	2	26·0 ±1·41	2	47·5 ± 6·40

* One sample = 40–45 mg of bifected adrenals.
† As cortisone.

effect, if ever there is one, on the performance of the glands to synthetize hormonal steroids. Moreover a striking decrease of steroid production is apparently displayed by adrenals of longer treated rats.

(2) When ACTH was added to adrenal slices obtained from glands of morphinized rats the stimulatory effects of the hormone on corticosteroid production appeared to be lessened. As a matter of fact we have detected in morphinized rats a failure of the ACTH injection to produce the expected increase of urinary steroids.

(3) Table 2 shows that morphine inhibits the 3-beta-ol-dehydrogenase activity of whole adrenal and testicle homogenates when added to the medium at concentration of 50–100 μg/g tissue.

TABLE 2. EFFECT OF MORPHINE ON ACTIVITY OF 3-β-OL DEHYDROGENASE OF RAT ADRENALS AND TESTICLES

Gland	Weight tissue g	Morphine μg/g	$\alpha:\beta$-Unsat. ketone (μmoles/g per hr) (average)	% Decrement under morphine
adrenal	0·05	—	22·0	
id.	id.	50	8·0	63·6
id	id.	100	4·8	78·2
testes	2	—	3·5	
id.	id.	50	2·0	42·1
id.	id.	100	1·3	62·9

(4) Table 3 depicts the liver performance to conjugate corticosteroids when hepatic slices excised from normal and morphinized rats are incubated in presence of DOC. The rate of DOC conjugation by liver slices from morphinized rats appears to be reduced when compared to controls.

TABLE 3. INHIBITORY EFFECT OF MORPHINE ON *in vitro* CONJUGATION OF DOC BY LIVER SLICES FROM TOLERANT RATS (MORPHINE 20 MG/KG DAILY FOR 30 DAYS)

No of samples	Rate of conjugation (μmoles/hr per g)	% of inhibition by morphine
Controls, 10	75	—
Tolerant rats, 10	12	84

DISCUSSION

In a previous report[1] it was shown that repetitive treatment with stabilizing doses of morphine induces in rats a decrease of urinary excretion of hydroxysteroids, aldosterone and 17-ketosteroids. It has been also referred that ACTH injection and exposure to cold resulted in a remarkable lesser adrenal response.

Apart from the possibility that the impaire ment of liver conjugation presented here may result in a decrease of urinary excretion of less soluble steroids, the present report demonstrates that morphine may display a direct influence on the hormonal metabolism of the adrenocortical tissue. Indeed, adrenal slices obtained from glands of properly morphinized rats produce corticosteroids at a very much lower degree than the controls. According to our previous observations on guinea pigs,[7] morphine exerted an inhibitory effect on steroid production even when added *in vitro* at various concentrations to rat adrenal and testicle homogenates.

Moreover ACTH added to incubation medium fails to increase the synthesis of hormones by adrenal slices obtained from morphinized rats. This observation attracts attention to the question of the steroid metabolic pathway undergoing morphine inhibition in adrenals.

Actually Samuels preparation for 3-β-ol-dehydrogenase system appears to be inhibited by morphine; it is not known, however, if this inhibition occurs in living animals, or if the drug may act on other enzymatic chains in adrenal and testicle.

Present results indicate that morphine may affect the endocrine functions much more than is expected. The question of the effect on these secretions by morphine, as due to a nonspecific mechanism, appears largely to be reviewed. The assessment of the pharmacological value of the property of morphine to influence steroid metabolism requires further investigations.

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THE BINDING OF AROMATIC SULFONAMIDES TO ERYTHROCYTES*

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Abstract—Earlier work *in vivo* showing that sulfanilamide and acetazolamide had an affinity for erythrocytes was confirmed and extended. Eighteen sulfonamides were studied *in vitro*. Twelve unsubstituted drugs (aryl-SO_2NH_2), all carbonic anhydrase inhibitors, had varying degrees of affinity for red cells; dissociation constants varied 10,000-fold. Six substituted drugs (aryl-SO_2NHR), all antibacterial agents and not carbonic anhydrase inhibitors, had no affinity for erythrocytes. Acetazolamide and the other unsubstituted drugs accumulated in canine red cells to 32–92 μmoles/l against concentration gradients. Sulfanilamide and other benzene-sulfonamides could be washed out readily, but acetazolamide and other heterocyclic compounds could not. The K_{diss} of the sulfanilamide complex is 2×10^{-4} M; that of the acetazolamide complex is about 10^{-8} M. Acetazolamide interferes competitively with the accumulation of sulfanilamide in red cells. Formation of the acetazolamide–red cell complex is not dependent on an energy source, pH, or temperature. Red cells of bird, reptile and fish also bind acetazolamide. Binding is to components within the cells, probably not hemoglobin. One component of binding is carbonic anhydrase; for most arylsulfonamides there are also other receptors, in both dog and man. The total K_{diss}. and the K_I for carbonic anhydrase are within one log unit of each other, for a given drug, in the four cases studied. The drug receptors in red cells have a "fit" similar to carbonic anhydrase since they react quite specifically with aryl-SO_2NH_2. In other tissues, such as lens, acetazolamide binds to carbonic anhydrase alone.

Apart from the bound fraction most of the unsubstituted arylsulfonamides appear in erythrocytes, both *in vivo* and *in vitro*, in diffusible forms. The degree of diffusion varies greatly among the drugs. The diffusible or free tissue concentration of drug, and its K_{diss}. or K_I, quantitatively determine the degree of binding or enzyme inhibition.

STUDIES of the pharmacology of acetazolamide in rats, dogs, and man[1, 2] showed it to have an unusual affinity for erythrocytes. Following oral or parenteral administration there is a fairly rapid decline in plasma concentration (half-life in dog and man about 100 min), but when the plasma concentration of drug is no longer detectable (<1 μg/ml), from 15 to 30 μg/ml are found in washed red cells. Thereafter, this value slowly declines; the half-life of acetazolamide in human erythrocytes *in vivo* is about 3 days.[2] A number of other compounds related in various ways to acetazolamide, all of which were carbonic anhydrase inhibitors of potency within about a 100-fold range of acetazolamide, showed qualitatively similar behavior *in vivo*.[3]

These observations appeared to be related to earlier work with sulfanilamide and its derivatives in which it had been found that sulfanilamide and N^4-acetylsulfanil-

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amide showed (both *in vitro* and *in vivo*) a much higher (red cell)/plasma ratio than the N^1-substituted sulfanilamides, such as sulfapyridine, sulfadiazine and sulfathiazole.[4–6]

Thus, it appeared that the binding to red cells in this series of compounds might have the same structural basis as that for carbonic anhydrase inhibition, i.e. $XR—SO_2NH_2$, where R is any homocyclic or heterocyclic ring and X may be any other substituent(s) of the ring (see Maren *et al.*[1] for discussion). But while there is a high concentration of carbonic anhydrase in mammalian (and avian) erythrocytes (about 30 μmoles/l.[9]), there was only circumstantial evidence in favor of the suggestion[10, 11] that it is this enzyme which binds acetazolamide to the red cell.

The following types of experiments have been done in an exploration of red cell binding of sulfonamides. (1) Blood concentrations in dog, following the administration of three representative drugs. (2) Addition of substituted and unsubstituted sulfonamides to dog and human blood, *in vitro*. (3) Effects of time and temperature variation on binding. (4) Competition between acetazolamide and sulfanilamide for binding site. (5) Uptake of acetazolamide by various red cell and lens preparations; effect of metabolic changes. (6) Binding of acetazolamide to non-mammalian red cells. (7) Dissociation constants of drug–red cell complex.

MATERIALS AND METHODS

Drugs were obtained as pure crystals or as powder, in their acid form, from the American Cyanamid Company, with the following exceptions: sulfacetamide was obtained from Schering Corporation; dichlorphenamide and chlorothiazide from Merck Sharp and Dohme; ethoxzolamide from Upjohn; and sulfisoxazole from Hoffman-La Roche. Primary solutions were made by the addition of 1 — 1·6 moles of NaOH per mole of drug. Subsequent dilutions were made in a 0·9 per cent solution of sodium chloride (hereafter referred to as saline).

Analyses for sulfanilamide, N^1-acetylsulfanilamide, sulfadiazine, sulfacetamide, N^1-ethylsulfanilamide, sulfamethoxypyridazine, sulfisoxazole, sulfaethylthiadiazole and chlorothiazide were done on 0·1 — 0·4 ml of fluid by the method of Bratton and Marshall[12]. This procedure measures the arylamino moiety. For chlorothiazide, the trichloracetic acid filtrate (2 ml) was treated with 1 ml of 3 N sodium hydroxide and heated for from 15 to 30 min at 100 °C. Following neutralization, the usual procedure was carried out. Analyses for acetazolamide, methazolamide, ethoxzolamide, CL 13,580, CL, 11,366, CL 5343, CL 13,475, CL 5342, and dichlorophenamide were done by the method of Maren *et al.*[13]; this procedure measures carbonic anhydrase inhibition, presumably an expression of the free arylsulfonamide group. Activity of the compounds as carbonic anhydrase inhibitors was determined in the same way except that equilibrium was achieved between enzyme and inhibitor, and data were reported in terms of K_I.[9] pK values were determined by titration with 0·10 M sodium hydroxide using either a Beckman or a Photovolt pH-meter.

Recovery of drug added to blood was estimated for each experiment and may be calculated from columns (6), (7) and (8) of Table 1a. Hematocrit was from 45 to 50 per cent. It may be noted from these data that recovery is satisfactory except in the case of dichlorophenamide, in which case it was from 60 to 70 per cent.

Male beagles were used for studies *in vivo*. Drugs were injected intravenously. Blood was withdrawn from another vein and immediately centrifuged for 20 min at

850 × *g*, plasma was withdrawn for analysis, the buffy coat discarded, and a sample of unwashed red cells taken for analysis. Following this, the remaining red cells were washed and centrifuged three times with from 4 to 10 vols. of saline. The concentrations of drug reported for "unwashed cells", both *in vivo* and *in vitro*, have been corrected for the plasma (8 per cent) trapped in cells.

Whole blood studies

Freshly drawn, heparinized dog (beagle) blood was used, except when, as noted, other species were investigated. To 10 ml of whole blood in a 25-ml Erlenmeyer flask

TABLE 1b. BINDING OF UNSUBSTITUTED SULFONAMIDES TO HUMAN RED CELLS

Drug	Added (μmoles/l.)	Plasma	R.B.C.	Red cells after (W_1	W_2	W_3 (μg/ml)	Drug–receptor complex (μmoles/l.)
Acetazolamide	100	21	19	18	17	17	not saturated
	200	44	40	37	35	35	158
Methazolamide	100	4	40	—	—	34	144
	200	57	52	44	38	38	161
Ethoxzolamide	100	1	26	26	22	22	not saturated
	200	47	47	42	34	36	140

Same procedure as for Table 1a.

was added 0·1 ml of solution of drug, in the appropriate concentration; the drug solution usually contained from 5 to 20 μmoles/ml, in saline; thus, 0·5 — 2·0 μmoles of drug were added to 10 ml of blood. The charged flask was then shaken in horizontal motion on a Burrell Shaker (position 4) for different times and at different temperatures. At the indicated time, about 3 ml of blood were drawn off and immediately centrifuged at 850 × *g* for 20 min; the hematocrit was noted. Plasma was removed for analysis, the buffy coat drawn off and discarded, and a sample of red cells taken for washing and analysis. The red cells were washed and centrifuged three times with from six to eight times their volume of saline, unless noted specially. Data reported are taken from duplicate, and in many cases, triplicate experiments.

Washed cells

Packed cells were washed three times with ten times their volume of saline, then suspended in saline for 16 hr at 4 °C. The cells were removed and suspended, with shaking at 37 °C for 1 hr, in 15 vols. of isotonic saline containing acetazolamide in concentrations of either 11 μg/ml or 44 μg/ml. The mixture was then centrifuged and the cells were washed twice with 50 vols. of isotonic saline and taken for analysis. The final supernatant fluid was free of glucose.

Buffered cells

The preparation was as just described, except that the incubation was carried out in various isotonic phosphate buffers in place of saline.

Bicarbonate

Washed cells were made up, as described, in saline containing 11 μg of acetazolamide/ml. Sodium bicarbonate was added to give 33 m-moles of CO_2/l. and CO_2 was bubbled in to yield a pH of 7·4. The washing and analytical procedure were then carried out as described above for blood.

Iodoacetate

Potassium iodoacetate (10^{-4} M in final solution) was added to 10 ml of a washed cell suspension containing 0·7 ml red cells. After 1 hr, acetazolamide (111 μg) was added and the mixture incubated for 45 min at 37 °C. The cells were separated by centrifugation, washed, and taken for analysis as described above. *Lysed cells* were prepared by treating washed erythrocytes with $\frac{1}{4}$ vol. of anhydrous ether, shaking and centrifuging. The top layer of denatured stromata was removed. The resulting clear solution was dialyzed for 16 hr at 25 °C against saline containing 2 μg of either acetazolamide or sulfanilamide per ml. *Ghosts* were prepared by treating with an equal volume of distilled water, 50 ml of citrated human blood, obtained from the blood bank. The preparation was centrifuged for 20 min at 2520 $\times$ *g*, the supernatant fluid decanted, and the pellet of ghosts remaining washed five times until the rinsings were clear. The viscous pellet (3 ml) was dialyzed with stirring against 100 ml of a solution of acetazolamide in isotonic saline, 0·5 μg/ml, for 15 hr at room temperature.

Equilibrium experiments

(a) The lysed cell preparation following dialysis against sulfanilamide was used. A series of dialysis bags containing such cells saturated with sulfanilamide were suspended at 4° in 5, 10, 20, 50, 100 and 1000 ml of water. Twenty-four hours later the bag contents and solutions were analysed for drug, and the dissociation constant calculated. (b) For drugs with much lower equilibrium constants and thus lower concentration gradient for dialysis of free drug from the bag, it was felt that equilibrium might not be attained. In practice, the system just described for sulfanilamide was applied to acetazolamide, but even when the external solution was 150 l. no drug appeared to be lost from the bag. The red cells were then used as their own dialysis surface; accordingly, 2 ml of red cells containing either acetazolamide, or CL 13,580, or ethoxzolamide in bound form were added to 2 l. of saline, and gently stirred at 4 °C. At intervals, samples of the mixture were withdrawn and both the saline fraction and the centrifuged cells were analyzed.

Carboxyhemoglobin was prepared by passing CO into the lysed cell preparation until it turned bright cherry-red. This preparation in one bag, and the untreated lysed cells in another, were dialyzed against a solution of acetazolamide, 1 μg/ml of saline, for 18 hr at room temperature. *Crystalline hemoglobin* was prepared according to Drabkin[14]. Twenty-five ml of washed human red cells were lysed with $\frac{1}{4}$ vol. of anhydrous ether. The denatured stromata were removed by centrifugation. The clear hemoglobin solution was placed in a dialysis bag and dialyzed against 3 vols. of a saturated solution of ammonium sulfate at 6 °C until approximately 40 per cent by volume was precipitated in the bottom of the bag. The bag was removed and the clear solution above the precipitate was decanted. Crystals of ammonium sulfate were added slowly, while orthorhombic crystallization was viewed through the

microscope. When a crop of crystals had formed, the mixture was re-centrifuged, the supernatant fraction was decanted, and the crystals formed were re-suspended in 3 ml of distilled water. This mixture was dialyzed overnight against 50 vols. of saline at 6 °C. This solution, which was dark red and clear, and was rich in carbonic anhydrase activity (approximately 100 units/ml, as estimated by the changing pH method[13]) was dialyzed against 2 μg of acetazolamide per ml of saline for 18 hr at room temperature.

A hemoglobin-free extract of human red cells was prepared according to Meldrum and Roughton[15], who used the method to concentrate and extract carbonic anhydrase from erythrocytes. It was dialyzed for 10 hr at 25 °C with stirring against a saline solution of acetazolamide containing 0·5 μg/ml.

Lens preparation

Lenses were obtained from either rabbit or dog, thoroughly minced with an equal volume of saline, and placed in a dialysis bag. Fresh dog lens had 309 units carbonic anhydrase per gram, in good agreement with data obtained earlier with rabbit lens.[16] This level of enzyme activity may be compared to 500 — 600 units/ml for dog blood. Lens was dialyzed for from 8 to 20 hr at 25 °C against solutions of various concentrations of acetazolamide. By conducting a control dialysis against saline, it was found that the lens lost about half its carbonic anhydrase activity during the procedure.

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RESULTS

1. *Blood concentrations*, in vivo

Figs. 1, 2 and 3 show the distribution of three sulfonamides in plasma and erythrocytes following their injection in the dog. Some of these data are available from the literature, but no studies appear to have been made on washed cells, or on the "diffusible component" in red cells. These data are presented in full, as a way of introducing the problem; each of the three drugs appears to be in a separate category, and representative of others we have studied.

Acetazolamide (*Fig.* 1). Two doses were studied, 5 and 50 mg/kg. Following the low dose (Fig. 1a), 1 hr was required for the maximum concentration of the drug to be attained in red cells; this lag was absent at the high dose (Fig. 1b). It is evident that the component bound to red cells is strongly fixed, as compared to plasma. The amount of drug initially bound is limited, being about 30 μg/ml from very high plasma concentrations (Fig. 1b) and 15 — 20 μg/ml from low plasma concentrations (Fig. 1a). Repeated washing *in vitro* does not decrease these figures. Thus, in a typical experiment, the red cells of the 4-hr sample of Fig. 1(a) were washed repeatedly with from seven to twenty times their volume of saline. Successive values (in μg/ml of red cells) were 16·7 (unwashed), 15·3, 16·6, 15·3, and 14·1. The diffusible component of drug in red cells was evident in the 1-min sample, and its concentration was greatest in the 4-min sample. Thereafter, the concentration of the diffusible component declined more or less parallel to that of drug in plasma. The magnitude of the diffusible component suggests that it may be in equilibrium with unbound drug in plasma, or possibly with the unbound un-ionized fraction, which is about one-third of the plasma concentration.*

* Plasma binding in this species, originally reported as 30 per cent,[1] is from 30 to 60 per cent in our current experiments; the un-ionized fraction is 65 per cent.[17]

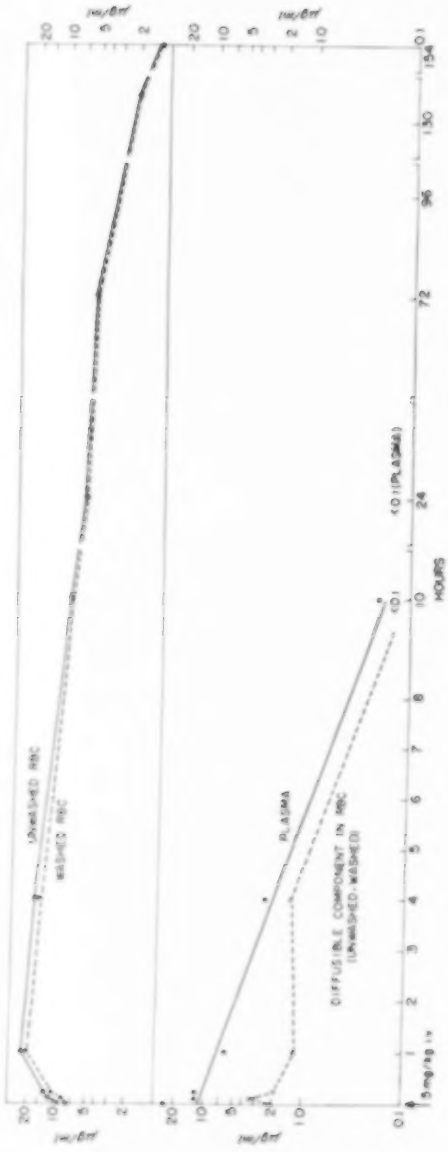

FIG. 1(a). Bound and diffusible components of acetazolamide in red cells: 5 mg/kg intravenously in dog.

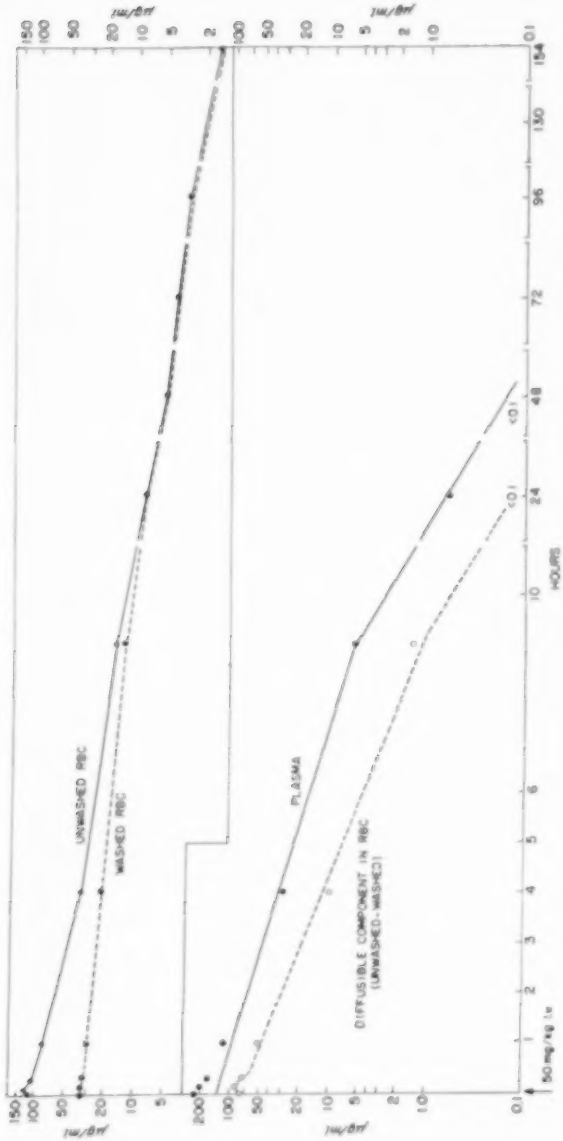

FIG. 1(b). Bound and diffusible components of acetazolamide in red cells: 50 mg/kg intravenously in dog. Three fractions of blood were analyzed at each period: unwashed erythrocytes, washed erythrocytes, and plasma. The "diffusible component" is the difference between unwashed and washed cells.

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Fig. 1(c) shows a further characteristic of the red cell binding of acetazolamide, namely, that more than one component seems to be involved. There are two decay slopes, one for the first 12 hr, and one thereafter. The first has a half-life of about 12 hr; the second of about 3 days. The first component is associated with measurable plasma concentrations of drug; the second component still exists when plasma concentrations are less than 0·1 μg/ml. After the first 12 hr, curves for high and low dose

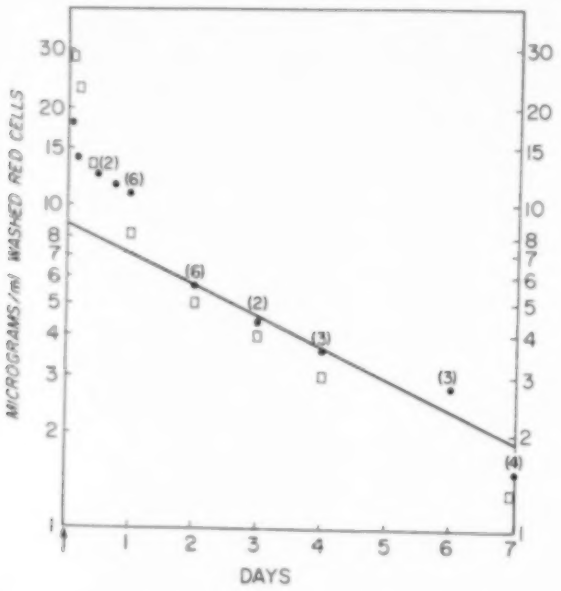

FIG. 1(c). Bound and diffusible components of acetazolamide in red cells: ● Gives average data for three-times washed red cells and number (*n*) of dogs following 5 mg/kg, given intravenously at arrow. Line is least square fit for decay from 2 to 7 days. □ shows expt. of Fig. 1(b), 50 mg/kg, given intravenously at arrow.

are indistinguishable. The same relationship was found when the blood concentrations of acetazolamide were followed in man.[2] Both (or all) of these components are tightly bound, since ordinary washing does not remove them. Analysis of the decay of acetazolamide *in vivo* (see Discussion) is relatively uncomplicated, since the drug is not metabolized in man and only to a minor degree in the dog.[1]

Sulfanilamide (*Fig.* 2). As with acetazolamide, there is evidence that sulfanilamide has an affinity for red cells. After the standard washing technique some drug remains in red cells. This, however, does not persist to the extent observed with acetazolamide. Rather, it declines in parallel fashion with the plasma concentration and is influenced by further washing *in vitro*. In a typical experiment, the red cells of the 4-hr point of Fig. 2(a) (10 mg/kg) were washed repeatedly with from seven to twenty times their volume of saline. Successive values, in micrograms of sulfanilamide per milliliter of packed cells, were 17·2 (unwashed), 8·5, 7, 4·2 and 1.

A particular characteristic of sulfanilamide, both *in vivo* and *in vitro* (Table 3), is that no matter how high the plasma concentration is, the red cell concentration

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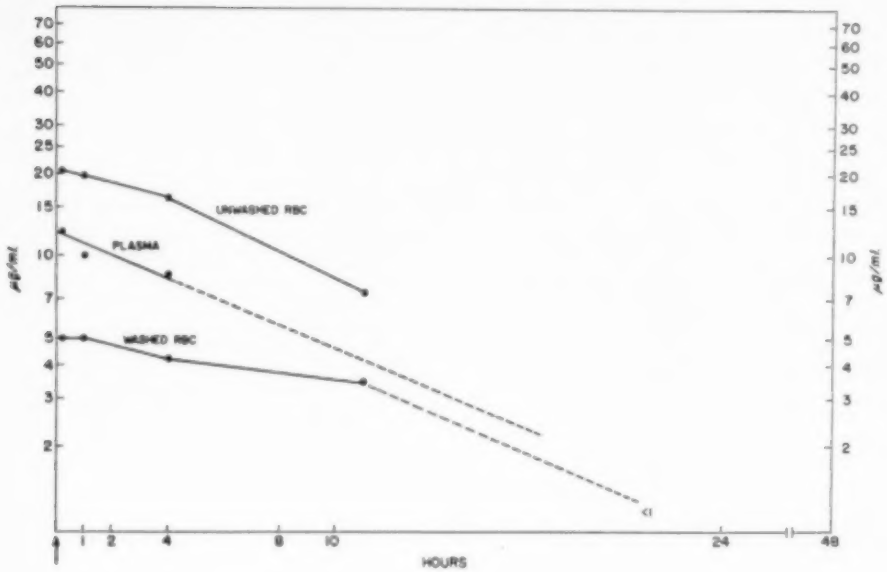

FIG. 2(a)

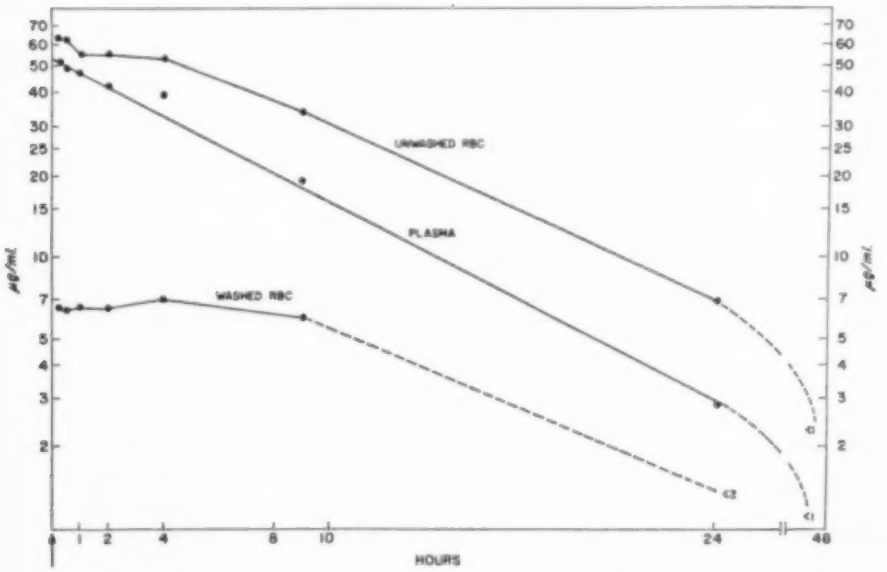

FIG. 2(b)

FIG. 2. Bound and diffusible components of sulfanilamide in red cells: (a) 10 mg/kg intravenously in dog; (b) 50 mg/kg intravenously in dog. See legend for Fig. 1.

exceeds it. The same may be true of N^4-acetylsulfanilamide; the data for these drugs suggest an additional loose reversible binding, perhaps (because of the very large capacity) to hemoglobin.

Sulfadiazine (*Fig.* 3). The data show that some sulfadiazine gains entrance into red cells, but this is entirely removed by washing. Supplementary tests showed that in the samples of Fig. 3, *all* of the drug was removed on the first washing of the three that were done routinely in these experiments.

The data of this section show three patterns of distribution in the blood for sulfonamide drugs. Acetazolamide is firmly bound to red cells, but with a diffusible

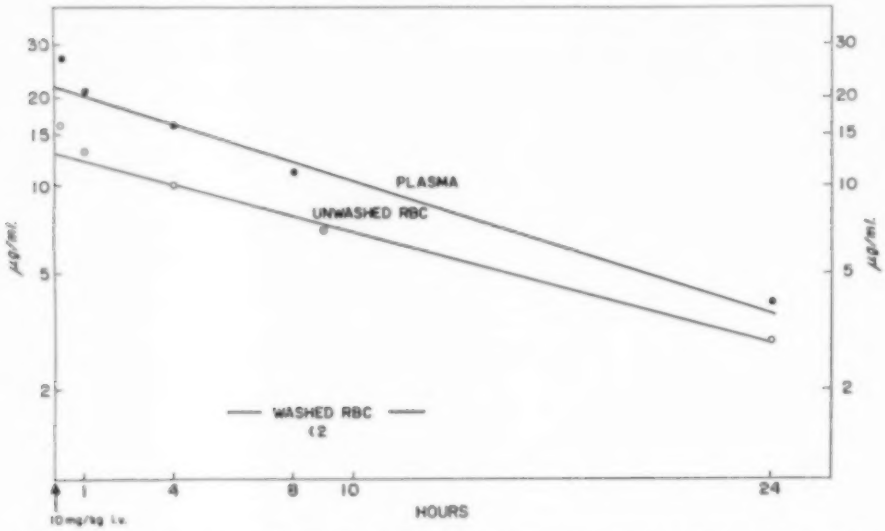

FIG. 3. Lack of binding of sulfadiazine (10 mg/kg given intravenously) to red cells in dog. Procedure as for Fig. 1 and 2.

component also; sulfanilamide accumulates in red cells but is not firmly bound; sulfadiazine has no affinity for red cells. The remainder of this study is an extension of these data to other drugs, and an exploration of the characteristics of the binding phenomenon.

2. *Addition of sulfonamides to blood*, in vitro

Tables 1a, 1b and 2 show the results of addition of eighteen drugs to whole blood. The twelve drugs listed in Table 1a are unsubstituted sulfonamides and have carbonic anhydrase inhibitory activity, as noted in column (5). Drugs of Table 2 (substituted sulfonamides) have no action against the enzyme. All measurements of red cell binding were made after incubation for 1 hr at 37 °C; representative drugs, run at different times, showed no changes in red cell uptake between ½ and 3 hr (Section 3). The variability may be judged by five replicate tests, each for two amounts of acetazolamide added to 10 ml of whole blood. After addition of 0·5 μmoles, plasma concentrations ranged from 4 to 7 μg/ml (av. 6·0), while red cell concentrations ranged from 11 to 14 μg/ml (av. 12). After addition of 2·0 μmoles, the plasma concentrations ranged

from 52 to 60 μg/ml (av. 55), while those of washed red cells were 18 — 19 μg/ml (av. 19). Variations for other drugs were of similar magnitude.

Table 1a shows that all twelve unsubstituted sulfonamides bind to canine red cells; Table 1b shows the same for three of these drugs, using human red cells. Binding is illustrated in two ways. For all but chlorothiazide and dichlorphenamide, drug was shown to move from plasma into red cells against a concentration gradient; and there is partial or complete resistance to washout of drug from the red cells.

There are notable differences among these twelve drugs, although the element of binding to red cells is common to all. There are at least three characteristics of binding, for each drug: (a) The amount of "diffusible component", i.e. the drug which diffuses in is not bound, and is largely or entirely washed out by the first saline treatment. This is measured arbitarily by the difference between concentration of drug in unwashed cells and that following the second wash; where binding is relatively weak, as judged by continuous washout at low concentrations (e.g. sulfanilamide), this is not accurately measurable by this technique. (b) The absolute amount bound, which is indicated by the concentration following the second and third wash. (c) The stability or dissociability of the drug–red cell complex, which is indicated in qualitative terms by the degree of washout in the successive steps. These three modalities appear to be independent of one another.

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(a) A sizable diffusible component (difference between columns (8) and (10) of Table 1a) is evident for ten of the twelve drugs when plasma concentrations are relatively high. Under these conditions (1-hr exposure at 37 °C) a certain quantity of drug appears to penetrate the cell by following a concentration gradient; the degree of diffusion is a characteristic of each drug, and possibly dependent on ionization and plasma binding. Two drugs (CL 13,580 and CL 11,366) showed a small or negligible diffusible component within the cell, at high plasma concentrations. These are ionized at body pH, and largely bound to plasma proteins.[18] Chlorothiazide does not enter or bind to cells from a moderate plasma concentration, (11 μg/ml); in this respect, it is unlike all the other compounds. There is diffusion and binding from higher plasma concentrations. With one exception the other nine drugs are less dissociated at pH 7·4; in part, this may explain their greater diffusion. The exception is CL 13,475, which may have special properties, since it is an N^1-substituted sulfanilamide.* The nine diffusible drugs have relatively low protein binding.[18] The diffusible component of acetazolamide (plasma binding from 30 to 60 per cent in the dog) is shown in Fig. 4. It is evident that this drug diffuses along a concentration gradient from plasma to free drug in cell water. The latter fraction washes out readily, leaving the bound component. This situation appears quite analogous to that *in vivo*, when plasma and washed cells are compared (Fig. 1a).

(b) The absolute amount bound to red cells was different among the different drugs. This was not related to potency as a carbonic anhydrase inhibitor, or to any evident structural characteristic. Under these conditions the amount bound was largely, but not entirely, independent of the plasma concentration, a tenfold increment in plasma concentration caused less than a twofold increment in that of red cells. This is precisely the situation observed *in vivo* with acetazolamide (Fig. 1).

* With some exceptions, the N^1-substituted sulfonamides generally diffuse into red cells, albeit to markedly varying degree (Table 2, and Frisk[8] and Fisher *et al.*[7]).

TABLE 1a. BINDING OF UNSUBSTITUTED SULFONAMIDES TO CANINE RED CELLS

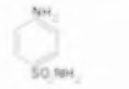
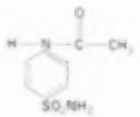
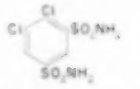
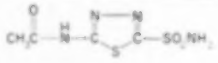
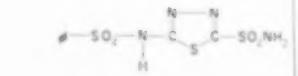
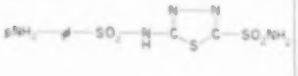
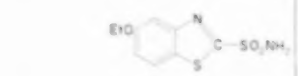
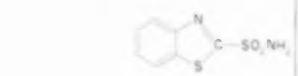
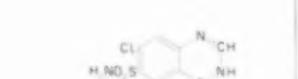
1	2	3	4	5	6	7	8	9	10	11	12
Structure	Name or number	M.W.	pK_a	K_I ($M \times 10^7$)	Drug added (μmoles/l.)	Concentration of drug (μg/ml) in Plasma	Red blood cells R.B.C.	after Wash 1	Wash 2	Wash 3	Drug–receptor complex (RI) (μmoles/l.)
	Sulfanilamide	172	10·4	28	20	3	6	3	2	1	
					50	8	13	7	4	2	
					200	28	39	15	8	4	—
					400	60	74	28	12	5	
					2000	304	364	89	19	7	
	N^4 acetylsulfanilamide	214	9·7, 10·4	5	50	5	16	11	9	8	37
					200	34	43	22	16	9	
	Dichlorphenamide	305	8·3, 9·1	0·25	50	9	9	8	6	5	—
					200	67	31	18	13	11	
	Acetazolamide	222	7·4, 9·1	0·08	20	1	8	8	8	8	54
					50	6	14	12	12	12	85
					200	55	45	31	23	19	
	CL 5343	180	7·8, 10·4	0·60	50	6	16	14	14	13	72
					200	36	36	27	21	19	105
	Methazolamide	236	7·2	0·09	50	12	14	9	9	9	
					200	58	27	13	9	9	38

TABLE 1a—*continued.*

1	2	3	4	5	6	7	8	9	10	11	12
Structure	Name or number	M.W.	pK_a	K_I ($M \times 10^7$)	Drug added (μmoles/l.)	Concentration of drug (μg/ml) in Plasma	R.B.C.	Red blood cells after Wash 1	Wash 2	Wash 3	Drug–receptor complex (RI) (μmoles/l.)
	CL 11,366	330	3·2, 9·0	0·01	50 200	14 116	17 33	18 32	16 31	14 30	43 91
	CL 13,475	345	4·2, 9·0	0·02	50 200	15 77	21 57	21 36	21 35	21 32	61 92
	CL 13,580	276	6·6, 8·8	0·02	50 200	11 80	18 21	17 18	17 18	17 18	63
	Ethoxzolamide	258	8·1	0·01	20 50 200	0·1 13 60	8 10 23	7 9 11	7 9 10	7 9 9	32
	CL 5342	214	7·8	0·01	50 200	7 40	11 23	9 14	8 10	8 9	37
	Chlorothiazide	296	6·7, 9·5	22	20 50 200	11 24 82	<4 8 20	<4 7 13	<4 6 13	<4 6 11	37

* All drugs added in 0·1 ml to 10 ml whole dog blood, and incubated 1 hr at 37 °C.

The final binding figure, designated (*RI*), is the fraction which appears insusceptible to further washing (Table 1a, column (12)). The data suggest that differences between experiments using high and those using low plasma concentrations, with four of the drugs, are largely due to limitation of diffusion or uptake from the low side. Five of the drugs gave the same value, whether approached from the high or the low side. For sulfanilamide, the present technique does not establish a figure for (*RI*), since the bond(s) are so weak that washout appears continuous. For dichlorphenamide, there was evidence that some drug is destroyed during incubation with red cells; the data are approximations only. For chlorothiazide, data are entered only from experiments with high plasma concentration, since there is clearly a major barrier to diffusion from the lower plasma concentrations.

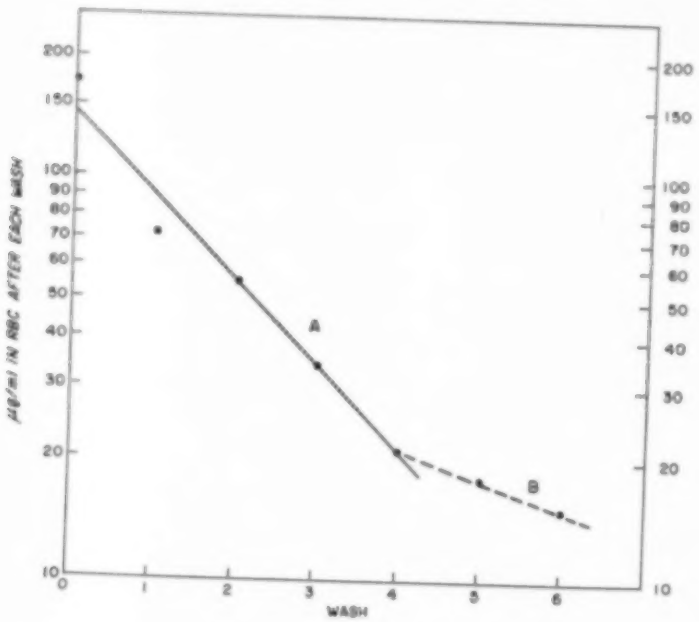


Fig. 4. Partial washout of acetazolamide from erythrocytes; 8 μmoles (1776 μg) of drug were added to 10 ml of whole blood of dog; original plasma concentration, 200 μg/ml. Same procedure as for Table 1a. A shows washout of the diffusible component; B shows the bound component.

Comparing the five drugs in which (*RI*) could be established with the greatest reliability, i.e. the same figure reached from the low or high side, there was evidence that there must be more than one binding site, since (*RI*) for one of these drugs (CL 13,580) was approximately twice that for the others. Data for the four drugs whose (*RI*) values varied somewhat for a single drug depending on the experimental design, also support the idea that the molar binding quantity may differ among drugs, by as much as threefold.

In addition to acetazolamide (Fig. 1), CL 13,580 and ethoxzolamide were studied *in vivo*, in order to compare their red cell binding with data obtained *in vitro*. One hour

following the intravenous injection in the dog, of 5 mg/kg, there were 16 μg of CL 13,580 per ml, and 9 μg of ethoxzolamide per ml, of washed red cells. These data agree closely with those of Table 1a. Further studies of these two drugs *in vivo*, in the manner shown in Figs. 1, 2 and 3 were not profitable in the present context, because these compounds are slowly metabolized in the dog. Thus, despite the extremely low dissociation constant of CL 13,580 and the red cell receptors (see Section 7 below), the concentration of drug in cells at 24 hr was 2·2 μg/ml, while that at 3 days was 0·1 μg/ml.

How carbonic anhydrase may be involved in this binding, and the relation between the external concentration and the amount bound, will be treated in Section 4 of the Discussion.

(c) The data of Table 1a show, by the degree to which the drugs are washed out from the red cells, the difference in "tightness" of binding. Eight of the drugs show no tendency toward washout of the bound fraction. It will be shown later that this would be expected under these conditions if the overall dissociation constant of drug–receptor complex (*RI*) is 10^{-7} M or less.

Table 1b shows experiments similar in design to those of Table 1a for three unsubstituted sulfonamides added to human blood. For each drug considerably more was bound to human than to canine red cells. For acetazolamide, the amount bound (44 μg/ml) agrees closely with that found *in vivo* for this species.[2] The carbonic anhydrase concentration of human and canine blood (or red cells) was found to be identical in this laboratory and by others.[19]

Table 2 shows that the N^1-substituted sulfanilamides are not bound to red cells, in agreement with previous work that has been cited. There is an all-or-none difference between these compounds and those of Table 1a and 1b; *all* of the unsubstituted drugs appear to be bound, whereas none of the substituted ones has this property.

3. *Effects of time and temperature*

Fig. 5 shows the effect of variations of time and temperature on the binding of both acetazolamide and sulfanilamide. When plasma concentration was high (2·0 μmoles added) essentially full binding of acetazolamide by cells was achieved in 1 hr at 37 °C, 24 °C or 9 °C. With lower plasma concentration, however (0·5 μmoles added), rate of binding appears somewhat dependent on temperature. In this case binding of acetazolamide did not change after 1 hr at 37 °C, but at the lower temperatures the rate was decreased, and saturation was not achieved after 3 hr at 9 °C. With sulfanilamide, neither time nor temperature appears to affect the phenomenon.

The data for acetazolamide suggest that the delay in establishment of binding equilibrium (Fig. 5a) is due to slowness of diffusion through the cell membrane, i.e. early in the experiment drug remains in plasma. There is no evidence that there is penetration of the cell, with a slow reaction between drug and receptor. Were this the case, drug would be washed out from the cells in the periods before 1 hr; this was not observed.

Sulfanilamide, which is generally more diffusible than acetazolamide (less ionized, less plasma binding), shows no comparable delay in getting into the cell, and reaching the binding site (Fig. 5(b)).

It appears from these data that the reaction between drug and red cell receptors may be extremely rapid, and is limited only by the diffusive characteristics of the drug.

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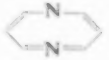
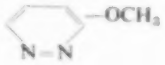
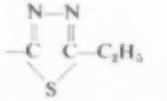
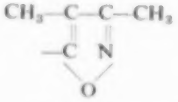
TABLE 2. LACK OF BINDING OF N^1 SUBSTITUTED SULFANILAMIDES TO RED CELLS *in vitro*

NH_2 / SO_2NHR — *R*	Name	M.W.	Plasma binding at 100 μg per ml % bound†	pK_a	μmoles drug added to 10 ml whole blood	Conc. drug found (μg/ml) Plasma	Unwashed* red cells	(Washed twice) red cells
$-C_2H_5$	N^1-ethyl sulfanilamide	200	3	10·7	0·5	16	3	<1
					2·0	41	36	1
$-COCH_3$	N^1-acetyl sulfanilamide (sulfacetamide)	214	16	5·4	0·5	14	6	<3
					2·0	49	30	2
	Sulfadiazine	272	17	6·5	0·5	22	5	<3
					2·0	71	38	<3
	sulfamethoxypyridazine	280	60	6·7	0·5	28	<2	<3
					2·0	88	16	<3
	sulfaethyl-thiadiazole	284	86	5·4	0·5	29	<2	<3
					2·0	105	<2	<3
	sulfisoxazole	268	68	5·8	0·5	24	1	<3
					2·0	87	13	<3

* By difference between total drug added and that found in plasma:

$$\frac{\text{drug added} - (\text{total drug in plasma} + \text{drug trapped in red cell plasma})}{\text{volume of red cells}}$$

It was estimated that 8 per cent of the volume of red cells was plasma.
In some cases, unwashed cells were analyzed directly.

† Kindly carried out by Dr. A. H. Anton, according to method of ref. 25.

4. *Competition between acetazolamide and sulfanilamide for red cell receptors*

Table 3 shows mutual interference of acetazolamide and sulfanilamide for the red cell receptors of the dog. Since no firm binding value for sulfanilamide could be established under these conditions (see also Table 1a), a quantitative estimate of the effect of acetazolamide on the sulfanilamide binding could not be made. However, it is evident that at equimolar concentrations of the two drugs, the binding of sulfanilamide is entirely eliminated by acetazolamide. At a ratio of sulfanilamide to acetazolamide of 40:1, the latter drug clearly interferes with binding of the former. At ratios

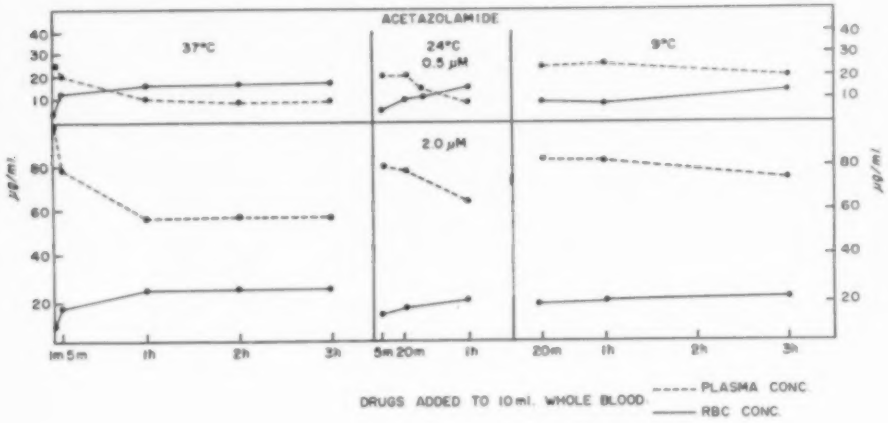


FIG. 5(a)

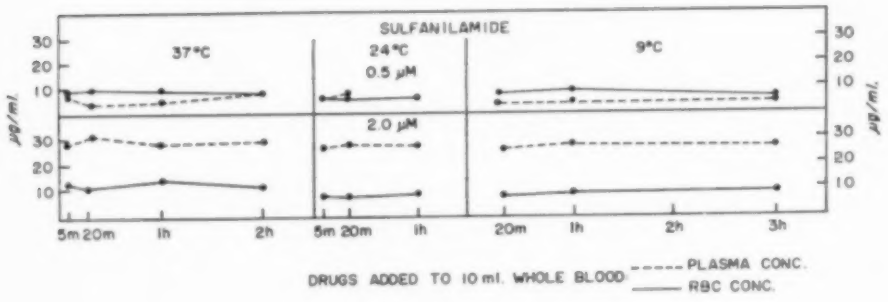


FIG. 5(b)

FIG. 5. Effect of time and temperature on red cell binding by sulfonamides *in vitro*. (a) acetazolamide; (b) sulfanilamide. In these experiments cells were washed twice with five times their volume of a 0·9 per cent solution of sodium chloride.

of 200:1 or greater, the binding of sulfanilamide was uninfluenced by acetazolamide. Using the binding of acetazolamide following the addition of 2 μmoles per 10 ml of blood as a criterion (19 μg/ml of red cells, see Table 1a), the effect of sulfanilamide may be estimated. The last column of Table 3 shows that 100 μmoles of sulfanilamide reduces the binding of acetazolamide by about 50 per cent. From these data, it may be judged that acetazolamide has about 100 times the affinity of sulfanilamide for the red cell receptors.

5. Uptake of acetazolamide by red cell and lens preparations

Table 4a shows representative experiments in which red cells were manipulated metabolically, or fractionated, and then tested for their ability to take up acetazolamide. Removal of glucose, alteration of pH or CO_2 equilibria and inhibition of respiration were without effect. Ghosts, which were found to contain no carbonic anhydrase, failed to take up drug; other cell fractions concentrated acetazolamide in

TABLE 3. MUTUAL INHIBITION OF BINDING TO RED CELLS BY ACETAZOLAMIDE AND SULFANILAMIDE

Sulf. (μmoles)	Acet. /10 ml)	Plasma	Red cell concentration after wash* μg sulfanilamide/ml				Acetazolamide bound μg/ml R.B.C. (W_3)
			Unwashed	W_1	W_2	W_3	
0·5	0	8	13	7	4	2	—
0·5	0·1	6	12	4	2	1	3·8
0·5	0·5	8	9	2	<2	<2	10·4
0·5	2·0	9	8	1	<2	<2	18·1
2·0	0	28	39	15	8	4	—
2·0	0·1	28	38	11	5	2	3·1
2·0	0·5	30	36	7	1	<2	10·3
2·0	2·0	31	38	4	<2	<2	16·6
20·0	0	304	364	89	19	7	—
20·0	0·1	300	364	76	15	5	1·7
20·0	0·5	304	364	61	9	2	6·4
20·0	2·0	304	364	68	8	1	11·2
100·0	0	1225	1562	360	71	19	—
100·0	0·1	1188	1475	355	75	13	0·9
100·0	0·5	1225	1475	355	57	18	4·0
100·0	2·0	1213	1475	365	62	10	9·2

* Added within 10 sec after sulfanilamide to whole heparinized dog blood.
All mixtures were incubated for 1 hr at 37 °C.

the usual fashion. The "Drabkin hemoglobin" fraction contains carbonic anhydrase; however, the role of hemoglobin as receptor was ruled out by the finding that the hemoglobin-free extract of Meldrum and Roughton[15] also took up drug.

Table 4b compares the reaction of acetazolamide with human red cells to that with lens, and indicates the role of carbonic anhydrase in the red cell binding phenomenon. In one type of experiment, equal volumes of suspensions of fresh minced dog lens and lysed red cells, each in a small dialysis bag, were suspended in a dilute solution of drug. The concentrations of cells and of lens were so adjusted that the enzyme content in the two bags was the same. Preliminary studies had indicated that, at the concentrations of acetazolamide used, diffusion was complete within 8 hr. After 24 hr, the outside solution and the contents of the bags were analyzed. The results showed six times as much uptake into red cells as into lens. Calculations were also made of the molar binding of drug, compared with the molar concentration of enzyme, as derived from kinetic data.[9] The data show that one mole of acetazolamide combines with one equivalent (or mole) of carbonic anhydrase in lens. In the red cell preparation, however, there was about ten times as much drug bound as could be accounted for by

the carbonic anhydrase concentration. The experiments with rabbit lens homogenate confirmed the fact that for this tissue, uptake of drug could be correlated directly with carbonic anhydrase concentration.

6. *Binding to non-mammalian red cells*

Acetazolamide was added to blood of the chicken, alligator (*A. mississipiensis*) and elasmobranch (*S. acanthias*). Results are shown in Table 5. It is evident that red

TABLE 4a. BINDING OF ACETAZOLAMIDE TO HUMAN RED CELL PREPARATIONS

I. *Washed and treated human red cells Incubated 1 hr at 37 °C*	Acetazolamide concentration (μg/ml)	
	Saline medium	Treated R.B.C.
Saline washed, glucose free	12	50
Saline washed, glucose free	64	57
Saline washed, glucose added (100 mg%)	15	49
Saline washed, glucose added (100 mg%)	60	48
Saline washed, 33 m-moles of HCO_3^- per l.: $pCO_2 = 58$	11	38
Saline washed, *K* iodoacetate added (10^{-4} M)	11	42
Phosphate-saline buffers pH 6·1	10	38
6·5	10	40
7·0	10	38
7·5	10	42
8·3	10	40
II. *Erythrocyte preparations in dialysis bag Incubated 25 °C for 18 hr*	Outside conc. in saline	Inside conc.
Lysed cells	2	19
Washed Ghosts	0·5	0·5
CO-treated,lysed cells	1	12
Drabkin hemoglobin	2	19
Meldrum–Roughton	0·5	13

I. Washed cells were suspended in 15 vols. of appropriate medium, containing drug.
II. Inside volume, approximately 2 ml; outside, 100 ml. See Methods sections.

TABLE 4b. BINDING OF ACETAZOLAMIDE TO LENS*

Preparation	Enzyme units/g	(E)† M × 10^{-7}	Drug conc. M × 10^{-7}		
			out	in	bound
Minced dog lens‡	89	15	18	31	13
Ether-lysed red cells, 1:6‡	95	16		192	176
Rabbit lens 1:1 homogenate	100	17	4	18	14
Rabbit lens 1:19 homogenate	10	1·7	1·3	3·4	2·1

* Lens or red cell preparations were put in dialysis bags (5 ml) surrounded by 100 ml of external solution, at 25 °C for 18 hr.
† From Maren *et al.*[9], 1 enzyme unit/g = 0·17 × 10^{-7} M.
‡ Suspended in same outside solution.

cells of all three vertebrate classes bind the drug, although in very different concentrations. The amount of drug bound is not related to the carbonic anhydrase activity of the blood.

7. Dissociation constants of drug–receptor complex

(a) Sulfanilamide. The lysed cell preparation saturated with drug was placed in dialysis bags and suspended in different volumes of water, as described in the Methods Section. In a typical experiment, initially there were 4·5 μg/ml bound to the lysed cells; at equilibrium (24 hr later), there were 2·6 μg/ml inside and 0·5 μg/ml in the

TABLE 5. BINDING OF ACETAZOLAMIDE TO BLOOD OF BIRD, ALLIGATOR AND FISH

	Acetazolamide added to 10 ml blood (μmoles)	Time (min.)	μg/ml		Hematocrit	Carbonic anhydrase units/ml blood*	Notes
			Plasma	washed† R.B.C.			
Chicken	0·5	20	5	27	26	200	Incubated at 37 °C
		110	4	27			
	2·0	20	38	45			
		110	33	44			
Alligator	0·5	20	16	3	27	30	Incubated at 23 °C. *A. mississippiensis* captured in Gainesville, Florida.
		120	13	4			
	2·0	20	45	7			
		120	42	8			
Dogfish	0·5	20	8	11	20	8	Incubated at 23 °C. These experiments were carried out on *S. acanthias* at Mt. Desert Island Biol. Lab. Salisbury Cove, Maine
		100	9	11			
	2·0	20	40	20			
		110	45	23			

* On this basis, dog and human blood (hematocrit = 45) have 600 units/ml.
† Twice with 5–7 vols. 0·9 per cent saline (chicken, alligator) or 1·5 per cent saline – 2 per cent urea (dogfish).

outside (5-ml) bath. Thus, if R represents the total receptor sites and RI the bound sites,

$$K_{diss.} = \frac{R - RI}{RI} \times (I_{free}).$$

The fraction $\frac{R - RI}{RI}$ is $\frac{4{\cdot}5 - 2{\cdot}1}{2{\cdot}1}$ or 1·14.

I_{free}, measured by the outside concentration, is $2{\cdot}9 \times 10^{-6}$ M, whence $K_{diss.} = 3{\cdot}3 \times 10^{-6}$ M.

Using the analytical data from the first four dialysis systems (from 5 to 50 ml outside volumes), a reciprocal plot of bound vs. free drug was made according to Klotz *et al.*[20]. A straight line was obtained; from its slope, the dissociation constant was calculated to be $2{\cdot}1 \times 10^{-6}$ M.

(b) Acetazolamide, CL 13,580, and ethoxzolamide. The system using red cells saturated with drug, suspended in saline, proved satisfactory. In a typical experiment, 2 ml of canine red cells containing 16·5 μg of acetazolamide per ml (corrected to 100 per cent hematocrit) were stirred gently in 2 l. of saline at 4 °C for 72 hr. Equilibrium was reached between 4 and 20 hr; thereafter, the concentration of drug in the red cells was constant at 13 μg/ml, and in the saline supernatant fraction, at 5 μg/l. or 10 μg

total.* This latter figure agrees reasonably well with the measured loss of drug from the red cells $(16{\cdot}5 - 13{\cdot}0) \times 2$ ml $= 7$ μg total. Thus, using in the equilibrium expression for I_{free} the average of these two independent measurements, 4·3 μg/l. or 2×10^{-8} M, the dissociation constant for acetazolamide and red cells is

$$K_{diss.} = \frac{16{\cdot}5 - 13}{13} \times 2 \times 10^{-8} = 5 \times 10^{-9} \text{ M}.$$

Four experiments of this type were done, and yielded results for $K_{diss.}$ ranging between 1 and 10×10^{-9} M.

Similar experiments were done with CL 13,580. In this case there was no measurable decrease in the concentration of drug in red cells; the saline contained at equilibrium 0·7 μg/l. This indicates that each ml of red cells had 0·7 μg less than at saturation; this difference would in fact be undetectable by comparison of red cells initially and at equilibrium. In the experiment cited, the intial saturation concentration was 16 μg/ml and, by difference, at equilibrium was 15·3 μg/ml. The dissociation expression is thus

$$K_{diss.} = \frac{16{\cdot}0 - 15{\cdot}3}{15{\cdot}3} \times 2{\cdot}6 \times 10^{-9} \text{ M} = 1 \times 10^{-10} \text{ M}.$$

Since we are measuring an overall dissociation constant, which appears to include several receptors, it was of interest to test further the possibility that there may be decisive differences among the binding constants of the different receptors. For this purpose, human blood was used, since it has a considerably larger binding capacity than canine blood, and conceivably a greater number of different receptors. Ethoxzolamide was selected, since its high activity makes analysis very sensitive, and since its K_I for carbonic anhydrase has been analyzed in detail.[9] At saturation, the red cells (again corrected to 100 per cent hematocrit) contained 48 μg/ml. Two ml of such cells were added to 2 l. of saline at 25 °C and the mixture was gently stirred. In this case, equilibrium was reached within 5 min; for the ensuing 24 hr the saline contained 6 μg/ml. The red cells, analyzed at 1 hr, contained 41 μg/ml. There was close agreement between the amount of drug found in the supernatant fluid (12 μg total) and the difference between drug bound to cells initially and at equilibrium (14 μg total). I_{free} may be taken as the average of these figures, 6·5 μg/l., whence the dissociation constant is

$$K_{diss.} = \frac{48 - 41{\cdot}5}{41{\cdot}5} \times 2{\cdot}3 \times 10^{-8} \text{ M} = 3{\cdot}6 \times 10^{-9} \text{ M}.$$

It is evident that most, if not all, of the receptors bind ethoxzolamide in very tight union, since no substantial number released drug under these conditions. The loss of drug to the free state is explicable on the grounds that the average drug–receptor complex has the dissociation constant cited above and that none of the receptors has a

* In this experiment, the saline supernatant fraction was reduced in volume 25-fold in a flash evaporator, in order to bring the concentration of acetazolamide into the detectable range. Appropriate controls were carried out to ensure that drug was recovered quantitatively following this procedure. It was necessary to use only 0·1 ml of concentrated saline in the analytical method to determine this amount of acetazolamide. The concentration procedure was not necessary in the cases of CL 13,580 and ethoxzolamide, which are more active inhibitors and thus detectable in smaller amounts.

markedly higher constant. Carbonic anhydrase, which may account for about one-fourth of these receptors (see Discussion), has approximately the same affinity for ethoxzolamide (Table 1a, column (5)) as the average receptor.

DISCUSSION

1. *Specificity of structures for red cell binding: degree of binding* (*Results, Sections* 1, 2 *and* 7)

Since all of twelve unsubstituted arylsulfonamides showed binding to red cells and none of six substituted ones was bound, it is tempting to state that this reaction is a general property of drugs in the class aryl-SO_2NH_2. Identical relations exist with respect to inhibition of carbonic anhydrase; all unsubstituted sulfonamides have activity at least as great as K_I of the order of 10^{-5} M, whereas substituted drugs are devoid of activity.[9]

Among these twelve drugs, variation was found in the molar concentrations bound, and, to a much greater degree, among the dissociation constants. With respect to the molar concentration, there was a threefold range in the same species (dog) between the lowest binding (ethoxzolamide; 32 μmoles/l.) and the highest (CL 13,475; 92 μmoles/l.). There was no obvious chemical or physical correlation among the drugs that bound relatively few sites, or those that bound larger numbers of sites. It was clear, however, that very closely related drugs had similar concentrations bound, i.e. the respective pairs: acetazolamide and CL 5343; ethoxzolamide and CL 5342; CL 11,366 and 13,475. With regard to the dissociation constants of the drug–red cell complex, in the four cases studied these showed an approximate range of 10^4, corresponding roughly to the order of inhibition-strength (as judged by the K_I, Table 1a) against carbonic anhydrase. The relationship between red cell binding and the carbonic anhydrase problem will be discussed in Section 3 below.

2. *Nature of the binding to red cells: diffusion* (*Results, Sections* 3, 4 *and* 5)

These data show that the binding of acetazolamide in erythrocytes is not energy-dependent, and it does not appear that "active transport" plays a part. Binding is not to hemoglobin, if acetazolamide is taken as a prototype (Table 4a). The fact that competition for the red cell receptors can be demonstrated between two quite dissimilar drugs of the aryl-SO_2NH_2 class (sulfanilamide and acetazolamide, Table 3) strongly suggests the common nature of the binding among the twelve drugs studied. Results of the competition experiment agree with data on individaul dissociation constants, in placing the relative affinity of acetazolamide to red cells at about 100 times that of sulfanilamide.

We regard the reaction as one of chemical bonding, possibly ionic in nature. On the other hand, diffusion plays an important role in determining the ultimate binding, both *in vivo* and *in vitro*. Certain drugs, such as sulfanilamide and methazolamide, diffuse readily into the red cell, and attain full binding from relatively low concentrations very rapidly. Acetazolamide occupies an intermediate position; full binding requires relatively high plasma concentration, and about 1 hr, both *in vitro* and *in vivo*. The acidic drug, CL 11,366, diffuses poorly. The data suggest that the actual binding may be instantaneous, but that, depending on the chemical type and the particular experimental conditions, the amount of diffusible drug available for binding is the limiting factor. Since this diffusible drug within the red cell (I_{free}) is the fraction

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also in equilibrium with carbonic anhydrase (or any other enzyme in cell water), these diffusive characteristics will have a bearing on the activity of such drugs as enzyme inhibitors, *in vivo*. This subject is treated in detail elsewhere.[18]

The diffusive characteristics of the substituted sulfonamides (Table 2) are of interest. Sulfaethylthiadiazole[6] shows very poor diffusion into red cells, parallel to low diffusion into cerebrospinal fluid.[21] Sulfisoxazole[21, 22] and sulfamethoxypyridazine[21–24] show moderate diffusion into red cells and cerebrospinal fluid.* Sulfadiazine, N^1-ethylsulfanilamide and sulfacetamide diffuse well into both red cells and cerebrospinal fluid.[6, 7, 21]

Both plasma binding and ionization limit diffusion. Sulfaethylthiadiazole is 99 per cent ionized and 86 per cent plasma bound. The most diffusible drug of Table 2, N^1-ethylsulfanilamide, is un-ionized at pH 7·4 and little bound. Comparison of two pairs of drugs with similar pK_a values, but different degrees of plasma binding (sulfacetamide and sulfaethylthiadiazole: sulfadiazine and sulfamethoxypyridazine) suggest that diffusion in this series is limited chiefly by plasma binding. The role of ionization in these compounds (pK_a 5·4 to 10·7) appears less critical. It may be that pK_a must be less than about 4 to play a decisive role—compare, for example, CL 11,366 and CL 13,475 in Table 1a. Lipid solubility does not appear to be a major factor: the chloroform partition coefficients of sulfamethoxypyridazine, sulfadiazine and sulfanilamide decrease in that order,[24] while their diffusibility into red cells increases.

3. *Relation of binding to carbonic anhydrase* (*Results, Sections* 5 *and* 6)

Since dog and human erythrocytes contain approximately 30 μmoles (or microequivalents) of carbonic anhydrase per l,[9] it is evident that in many of the present experiments, we are dealing with drug that is bound also to other receptors. There is no indication whether these receptors are on the carbonic anhydrase molecule or on other proteins.

In the data of Table 1a, obtained in dogs, five drugs show a binding concentration of from 32 to 38 μmoles/l. of red cells. It is reasonable to suppose that in these cases the observed binding is all, or nearly all, to the enzyme. It is significant, however, that two of these drugs (methazolamide and ethoxzolamide), when added to human blood (Table 1b), show binding to the extent of 161 and 140 μmoles/l., respectively. Clearly, in these cases most of the binding is not to carbonic anhydrase. Data of Table 1b and the dissociation constant of ethoxzolamide and human red cells (Section 7 above) show that the bond to the non-carbonic anhydrase receptors is generally of the same order of dissociation as that to the enzyme.

Table 1a shows that acetazolamide and four other drugs bind to canine red cells in amounts two to three times greater than that required for saturation of carbonic anhydrase.† Fig. 1(c) shows the same for acetazolamide *in vivo*; two separate decay curves of drug in red cells suggest different binding sites, each with a relatively low

* Sulfamethoxypyridazine achieves considerably higher concentration in cerebrospinal fluid of dog than of man. This appears due to moderate (from 55 to 60 per cent) plasma binding in the dog,[23–25] but high binding (from 80 to 90 per cent) in man.[25]

† The equivalent weight of carbonic anhydrase is based on a study of the inhibition of the catalyzed hydration of carbon dioxide, and agrees with the molecular weight of the enzyme.[9] There may be other binding sites on the enzyme, conceivably related to other reactions. Studies of the kinetics of inhibition of the dehydration of carbonic acid are under way in this laboratory by Dr. Kenneth C. Leibman.

dissociation constant. If the half-lives are taken as an index of the relative affinity for what appear to be two receptors in the red cells, one (data for the first day; half-life 12 hr) would have about six times higher dissociation constant than the other (line drawn for 2- to 7-day data; half-life 3 days). If these latter data are extrapolated back to zero time, the theoretical binding at saturation is 8·8 μg/ml of red cells, or 40 μmoles/l. It is suggested, therefore, that the binding observed in red cells after the second day is to carbonic anhydrase. Support for this idea comes from analysis of data on the pharmacology of acetazolamide in man.[2] In those studies, as in Table 1b, the initial uptake of drug by red cells was high; the 24-hr concentration *in vivo* averaged 32 μg/ml of washed red cells. However, data obtained in the period from 2 days to 2 weeks after drug administration yielded a decay curve for acetazolamide in human red cells which was extrapolated back to zero time to yield a much lower value, 11 μg/ml. Such a curve is similar to that of Fig. 1(c), despite the marked contrast in total binding of drug to red cells in the two species. But since the carbonic anhydrase activity of red cells in dog and man is the same, and in view of the similar drug–red cell decay curves after the first day, it appears that acetazolamide is equally bound to enzyme in the two species. We conclude that in red cells of both species, but to a larger extent in man, there are binding sites for acetazolamide other than carbonic anhydrase, with slightly less affinity for the drug. Such differences in affinity appear below the resolution of the physical methods by which we have measured dissociation or inhibition constants, but are manifest in the sixfold differences between the slopes of the drug decay from red cells on the first day (total binding sites) and that for subsequent days (enzyme site) in the data of Fig. 1(c) and Maren and Robinson[2].

Similarly, the data of Table 5 show that there are receptors other than carbonic anhydrase for acetazolamide in red cells of bird, reptile and fish.

4. *Dissociation constants and "washout". Concentrations of free drug necessary for binding or inhibition (Results, Sections 2 and 7)*

The washout of drug from red cells (Tables 1a and 1b) reflects the total dissociation constants ($K_{diss.}$) or in the cases where binding is only to enzyme, inhibition constants for carbonic anhydrase (K_I). Other factors which affect the final binding characteristics are diffusion properties of the drug, the concentration of free drug (I_{free}) at equilibrium, and the number of binding sites for the particular drug in the individual species. Examples are given to illustrate these points.

Suppose that the total equivalence of receptors is 100 μmoles/l. and sulfanilamide is used. Both the K_I and the $K_{diss.}$ is 3×10^{-6} M. The loose combination of sulfanilamide with a very high concentration of red cell receptors (see Results, Section 1 and Table 3) may be ignored. If drug is added, as shown in Table 1a, to make a total concentration in blood of 200 μmoles/l., which is assumed to diffuse readily into cell water,* the following will apply:

$$K_{diss.} = \frac{[(R) - (RI)] \times [(I) - (RI)]}{(RI)},$$

* Correction for plasma binding and water concentration of cells are small factors here and are neglected. Differences in the number of receptors over a fivefold range will not seriously influence the equilibria.

where in molar terms (R) is initial receptor sites, (I) is initial drug concentration and (RI) is bound sites. $(I_{free}) = ((I) - (RI))$. In units of 10^{-6} M, and rounding small fractions:

$$3 = \frac{(100 - 97) \times (200 - 97)}{97}.$$

$I_{free} = 103 \times 10^{-6}$ and the percentage of bound receptors is $RI/R \times 100 = 97$ per cent. Since sulfanilamide is diffusible and reasonably soluble, let us assume that each wash removes 80 per cent of free drug, whence three washes will leave 103 μmoles/l. $\times 0{\cdot}2^3 =$ 0·8 μmoles/l. Using a rearrangement of the above equilibrium expression, we may solve for per cent binding in terms of I_{free}:

$$\frac{RI}{R} \times 100 = \frac{(I_{free})}{(I_{free}) + K_I} \times 100 = \frac{0{\cdot}8}{0{\cdot}8 + 3} \times 100 = 21 \text{ per cent.}$$

In this model, washing would reduce binding very greatly; this was the finding for sulfanilamide (Table 1a). Similar calculations for initial (I) of 2000 μmoles/l. yields an initial binding of 99·8 per cent. In the above model, washing three times reduces this to 84 per cent. The data of Tables 1a and 3 confirm experimentally that only at exceedingly high I_{free} concentrations may binding be maintained with a drug of these K_I and solubility characteristics.

Chlorothiazide (Table 1a) has about the same K_I as sulfanilamide. Presumably, as for other drugs, $K_{diss.}$ is about the same as K_I. The initial equilibrium figures for sulfanilamide will then apply—97 per cent saturation of red cells when 200 μmoles are added per liter of blood. The experimental data show that this is achieved, but also show much less washout than for sulfanilamide. The data (binding from low side) show that this drug is poorly diffusible into cells, and we may suppose that the low washout is a consequence of this. If, in the experiment cited, washing is 50 per cent efficient (instead of 80 per cent, as suggested for sulfanilamide) I_{free} after the third wash will be 103 μmoles/l. $\times 0{\cdot}5^3 = 12{\cdot}5$ μmoles/l., whence

$$\frac{RI}{R} \times 100 = \frac{12{\cdot}5}{12{\cdot}5 + 3} \times 100 = 81 \text{ per cent.}$$

Only a small reduction in saturation is seen, even though the K_I or $K_{diss.}$ is the same as that for sulfanilamide.

Methazolamide has K_I of approximately 10^{-8} M, and, in the dog, the receptor is virtually all carbonic anhydrase. The data show that this compound is readily diffusible. Saturation is achieved and maintained from the low side, i.e. following addition of 50 μmoles/l. The receptor (enzyme) concentration is known with some accuracy, as 30 μmoles/l. Using the same conventions and assumptions as for sulfanilamide, the full initial equilibrium expression is

$$0{\cdot}01 = \frac{(30 - 29{\cdot}98) \times (50 - 29{\cdot}98)}{29{\cdot}98}.$$

$I_{free} = 20 \times 10^{-6}$ M and the percentage of bound receptors is

$$\frac{RI}{R} \times 100 = 99{\cdot}93 \text{ per cent.}$$

If washing is 80 per cent efficient and the usual three washes are carried out, I_{free} will be reduced to 20 μmoles/l. $\times$ $0{\cdot}2^3$ = $0{\cdot}16$ μmoles/l. Percentage binding is then

$$\frac{RI}{R} \times 100 = \frac{0{\cdot}16}{0{\cdot}16 + 0{\cdot}01} \times 100 = 94 \text{ per cent.}$$

Since the analytical error is from 5 to 10 per cent, this cannot be distinguished from the initial saturation value, Thus, washing would be expected to have no noticeable effect for drugs of this K_I (or $K_{diss.}$), even if diffusion and solubility are high. These are the findings of Tables 1a and 1b.

Acetazolamide, as would then be predicted, is not washed out. Lack of complete binding from the low side is attributed to inadequate diffusion.

Similarly, those drugs of low $K_{diss.}$ or K_I which are not metabolized *in vivo* (e.g. acetazolamide and methazolamide) persist in red cells and tissues containing carbonic anhydrase, in equilibrium with exceedingly low concentrations of drug in plasma. Such equilibria, and their relation to enzyme inhibition in the kidney and choroid plexus, will be considered elsewhere.[26]

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SHORT COMMUNICATIONS

Inhibition of catechol-O-methyl transferase by pyrogallol in the rat

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THE MAJOR route of metabolism of circulating catecholamines in the rat has been shown to involve meta-O-methylation by catechol-O-methyl transferase.[1] S-Adenosylmethionine is the methyl group donor, and the reaction requires a divalent cation.[2] Pyrogallol has recently been shown to be an inhibitor of this enzyme:[3, 4, 5] however the kinetics of this inhibitory action *in vitro* and the effectiveness of pyrogallol *in vivo* as an enzyme inhibitor in the tissues have not been studied in detail. This communication presents data which bear on these topics.

Optimal conditions for the assay of the enzyme were as follows: 2 μmoles of l-norepinephrine, 0·5 μmole of S-adenosylmethionine, 2 μmoles of magnesium chloride, 50 μmoles of sodium phosphate buffer (pH 7·9), and an appropriate amount of enzyme in a total volume of 1·0 ml. After incubation at 37 °C the reaction was stopped by the addition of 0·5 ml of 0·5 M sodium borate buffer (pH 10), and the mixture was assayed fluorimetrically for 3-methoxy norepinephrine.[2] The reaction rate was not altered by greater than optimal concentrations of substrate or S-adenosylmethionine, but magnesium concentrations above 5×10^{-3} M were inhibitory. Under optimal conditions the rate was linear with time for only 20 min. In previous studies of the kinetics of this enzyme, lower concentrations of S-adenosylmethionine and 30–90 min periods of incubation were used.[2, 6] In our system valid kinetic data could not be obtained under these conditions.

Catechol-O-methyl transferase was purified approximately 30-fold from the liver, brain, and heart of the rat, as described by Axelrod and Tomchick.[2] The rate of norepinephrine metabolism by these preparations is shown in Fig. 1. The K_m for norepinephrine was approximately 3×10^{-4} M, regardless of the source of the enzyme. This differs from previous reports that the K_m for epinephrine is $1{\cdot}2 \times 10^{-4}$ M for the liver enzyme,[2] but less than 10^{-5} M in brain.[6] The K_m for S-adenosylmethionine was found to be 4×10^{-5} M.

Pyrogallol is known to be a substrate for catechol-O-methyl transferase,[7] and its inhibitory action has been described as competitive.[3] As illustrated in Fig. 1, however, the inhibition of norepinephrine metabolism by pyrogallol appears in this system to be at least partially non-competitive. The degree of non-competitive inhibition was greatest in the most purified enzyme preparations and increased slightly if the enzyme was pre-incubated with pyrogallol and substrate before the addition of S-adenosylmethionine. In the experiment described in Fig. 1, a partially purified (30-fold) liver preparation and a 5-min pre-incubation period were employed. The K_i for pyrogallol was 8×10^{-6} M under these conditions.

The degree of enzyme inhibition produced by pyrogallol *in vivo* was estimated in various tissues of the rat at several time intervals following the intraperitoneal injection of the inhibitor. Tissues were homogenized in 1 vol. of cold 0·1 M sodium phosphate buffer (pH 7·9) containing 2·5 μmoles of magnesium chloride per ml. The homogenate was centrifuged at 15,000 **g** for 20 min, and the supernatant fluid containing the soluble

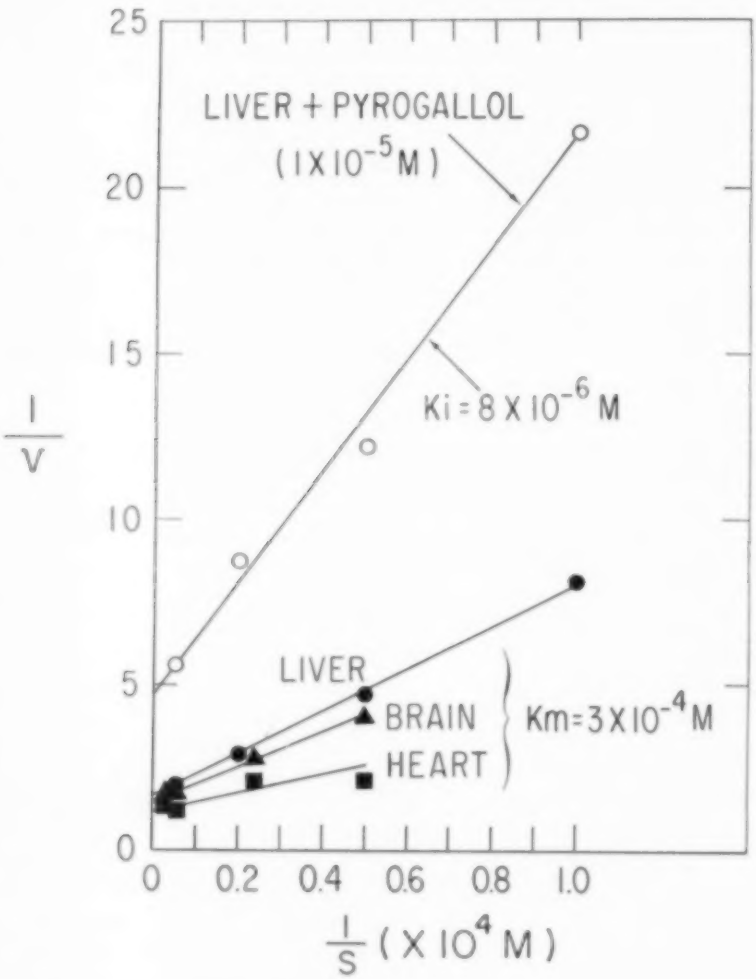

FIG. 1. Rate of norepinephrine metabolism *in vitro* by catechol-O-methyl transferase from three separate organs of rat; inhibition of liver enzyme by pyrogallol (data plotted by method of Lineweaver and Burk). Liver enzyme (0·48 mg protein) incubated 10 min; brain (1·8 mg protein) and heart (2·0 mg protein) preparations incubated 15 min; v is expressed as μ moles/hr per 1 mg protein for liver, per 15 mg protein for brain, and per 75 mg protein for heart, in order to fit all curves conveniently on one graph.

enzyme was removed for assay. Little or no activity was found in the particulate sediment. The incubation beaker contained 0·9 ml of the supernatant fluid, 2 μmoles of l-norepinephrine, and 0·5 μmole of S-adenosylmethionine in a volume of 1·0 ml. Dilution of the inhibitor from its concentration in tissue was thus maintained at a minimum. The percentage inhibition was determined by comparing the activity of tissues from pyrogallol-treated rats with that from control animals. In these concentrated supernatant solutions some activity (10–40 per cent of maximal) could be detected in control brain and heart without the addition of S-adenosylmethionine. The degree of inhibition was determined in the presence of both optimal concentrations and endogenous concentrations of co-factor (Table 1). The inhibitory effects of

TABLE 1. *In vivo*-INHIBITION OF CATECHOL-O-METHYL TRANSFERASE IN THE LIVER, BRAIN, AND HEART OF THE RAT FOLLOWING THE INTRAPERITONEAL INJECTION OF PYROGALLOL

Dose	Organ	Percentage Inhibition*					
		No added *S-adenosylmethionine*			With added *S-adenosylmethionine*		
		15 min	30 min	60 min	15 min	30 min	60 min
Single injection:							
50 mg/kg	Liver	—	—	—	78	41	17
	Brain	100	65	—	44	31	9
	Heart	100	70	—	48	32	3
200 mg/kg	Liver	—	—	—	—	96	84
	Brain	—	100	100	—	78	63
	Heart	—	100	100	—	81	73
Repeated injection:							
50 mg/kg per 30 min for 3 hr†	Liver	—	—	—	90	—	—
	Brain	100	—	—	62	—	—
50 mg/kg per 30 min for 16 hr†	Liver	100	—	—	90	—	—
	Brain	100	—	—	76	—	—
	Heart	100	—	—	67	—	—

* See text for details of procedure.

† Rats injected every 30 min and sacrificed 15 min after the last dose.

pyrogallol were of short duration *in vivo*, but effective inhibition of the enzyme could be achieved for 30–60 min with a single dose of 200 mg/kg. A repeated dose of 50 mg/kg every 30 min was required to maintain more prolonged enzyme inhibition.

These studies confirm that pyrogallol is a potent inhibitor of catechol-O-methyl transferase. Although this agent probably acts in large part as a competitive substrate, a non-competitive component of its action can be demonstrated *in vitro*. At adequate dosage levels pyrogallol also inhibits this enzyme *in vivo*. In separate studies reported

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elsewhere[8] the inhibition of catechol-O-methyl transferase with pyrogallol *in vivo* produced no accumulation of endogenous norepinephrine in the brain or heart of the rat.

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Improved preparation of 5-bromo-2′-deoxycytidine

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THE need for a large supply of 5-bromo 2′-deoxycytidine, as well as small amounts of it with suitable isotopic labels, for study in this laboratory as either a chemotherapeutic agent or one to reduce the threshold to radiation injury, has prompted us to re-examine the preparation of this compound. The method described by Frisch and Visser[1] appeared to be impractical for several reasons: first, the method involves the use of a special source of ultraviolet light and, therefore, quartz reaction vessels; second, glacial acetic acid as the solvent for bromination leads to the undesirable acetylation of the deoxyribose moiety, a circumstance which necessitates subsequent deacetylation by methanolic ammonia; third, purification of the product by the use of a column of Amberlite IR-120 (H^+ form) leads to extensive cleavage of 5-bromocytosine from the deoxyribonucleoside.

We have found that bromination of 2′-deoxycytidine can be conveniently and rapidly accomplished in pyridine; the yield of 5-bromo-2′-deoxycytidine is quantitative. When the crude 5-bromo-2′-deoxycytidine hydrobromide was purified by the use of a charcoal column, an 87 per cent yield of free 5-bromo-2′-deoxycytidine was obtained. The deoxyribonucleoside appears to be completely stable on the charcoal column and no formation of 5-bromocytosine was observed even after prolonged standing.

In one experiment, formamide was employed as the solvent for bromination, in a manner similar to that of an unpublished procedure of Markham, referred to by Bessman *et al.*[2], for the preparation of 5-bromo-2′-deoxycytidine triphosphate; the 5-bromo-2′-deoxycytidine thus formed was purified

according to the method of Frisch and Visser.[1] As mentioned above, some cleavage of the deoxyribonucleoside was observed under these conditions; accordingly, the products were further purified with the aid of a Dowex-1 formate column. The final yield was about 60 per cent.

In the preparation of 5-bromo-2′-deoxycytidine-H^3 from tritiated-2′-deoxycytidine on a micromole scale, the radioactive product was purified by the use of a Dowex-1 formate column. Prompt elution of the column was necessary in order to minimize cleavage of the deoxyribonucleoside. With different batches of the resin, the yield varied from 60 to 90 per cent. The loss of radioactivity, which resulted from the replacement of hydrogen by bromine in position 5, accounted for approximately one-half of the total H^3 of the 2′-deoxycytidine hydrochloride; this finding gives strong support to the hypothesis that the precursor was labeled exclusively in positions 5 and 6.

EXPERIMENTAL

5-Bromo-2′-deoxycytidine. (A) *Bromination in pyridine.* A mixture of 2′-deoxycytidine hydrochloride* (2·6 g; 0·01 mole) in pyridine (600 ml) was stirred at room temperature until partial solution of the compound was obtained. Of a solution of bromine in carbon tetrachloride (1 ml of bromine diluted with carbon tetrachloride to give a volume of 10 ml), 7 ml were added and stirring was continued at room temperature until the solution became entirely clear (30 min) (on the basis of ultraviolet absorption the bromination was nearly quantitative). The pyridine solution was concentrated at 50° *in vacuo* to dryness. The residue thus obtained was dissolved in water (70 ml) and the reaction was adjusted to pH 9 with a solution of sodium hydroxide (10 N). The solution was then put on a charcoal column†, 2·5 × 27 cm; this was washed with distilled water until free of bromide (7–10 l.). The product was eluted from the column with 95 per cent ethanol (6–8 l.) and the solvent was removed *in vacuo* at 50°. The residue crystallized to give 5-bromo-2′-deoxycytidine in over-all yield of about 87 per cent; m.p. 171–175° (reported 175–179°[1]); λ_{max}^{pH2} 299–300mμ (ε 9,600); $\lambda_{max}^{H_2O}$ 287 mμ (ε 5,880); $\lambda_{max}^{95\%\,EtOH}$ 290 mμ (ε 5,650); the product may be recrystallized from methanol and ethyl acetate. (B) *Bromination in formamide.* To a solution of 2′-deoxycytidine hydrochloride (1·3 g, 0·005 mole) in formamide (8 ml), a solution of bromine in carbon tetrachloride (4·4 ml; 1 ml of bromine diluted with carbon tetrachloride to give a volume of 10 ml) was added until there was a distinct light yellow color present due to a slight excess of bromine. Completion of bromination was indicated by the ultraviolet absorption spectrum of the reaction mixture, which should have a λ_{max}^{pH2} 299 mμ. The mixture was now taken up in water (200 ml), and purified as described in (A). A less pure product can be obtained by substituting, in place of charcoal, acidic resins such as Amberlite IR-120, according to the method of Frisch and Visser[1]; with this procedure the yield of the crude 5-bromo-2′-deoxycytidine was 60 per cent. This material was rechromatographed on a Dowex-1 formate column to separate it from unreacted 2′-deoxycytidine and 5-bromo-cytosine.

5-Bromo-2′-deoxycytidine-H^3. A solution of tritiated 2′-deoxycytidine‡ in 50 per cent ethanol (0·75 ml, 0·75 mc, 0·68 μmole) was reduced to dryness at 40° and vacuum dried at room temperature for 1½ hr. To the dried residue were added a solution of non-radioactive 2′-deoxycytidine hydrochloride in pyridine (0·27 ml; 3 mg/ml, 3·08 μmoles) sufficient to bring the specific activity of the final solution to 0·75 mc/3·76 μmoles (199 μc/μmole), and a solution of bromine in carbon tetrachloride (0·25 ml of a solution of bromine in carbon tetrachloride containing 0·1 ml per 100 ml). The solution was allowed to stand for 10 min and then evaporated to dryness at 40°, followed by brief drying under vacuum to remove the pyridine. The residue was taken up with water (1 ml); the solution was adjusted to about pH 11 with 1 N sodium hydroxide, divided into two equal portions, and put on two 30 × 1 cm Dowex-1 formate columns. These were subjected to gradient elution with water (1 l.), agitated

* Purchased from California Corp. for Biochemical Research, Los Angeles, Cal.

† Composed of activated carbon, grade 20 × 40, Atlas Powder Co., Wilmington, Delaware.

‡ Purchased from Schwarz Bio-Research, Inc., Mt. Vernon, New York, and presumed, from the method of preparation, to contain tritium only in positions 5 and 6; specific activity, 1100 μc/μmole.

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magnetically, into which 0·025 N formic acid was introduced dropwise in a continuous manner. The eluate was collected in 1-ml fractions; yield, 610 μg, specific activity, 92 μC/μmole.

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A single extraction method for the determination of both norepinephrine and serotonin in brain

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IN THE course of studies in this laboratory on the physiologic role of brain norepinephrine and serotonin and the effects of various drugs on their levels, separate extraction methods for the two amines were developed.[1, 2, 3] In these procedures the amines are extracted from brain homogenates into *n*-butanol: serotonin from an alkaline homogenate, and norepinephrine (because of its instability in alkali) from an acid homogenate. The discovery that serotonin can also be extracted into *n*-butanol from an acid homogenate has made it possible to use the unmodified norepinephrine extraction procedure of Shore and Olin[2] for the extraction of both amines.

With the single extraction procedure, it is possible to measure the amounts of each brain amine in separate aliquots of the final acid extract. Neither amine interferes with the determination of the

TABLE 1. RECOVERY OF SEROTONIN ADDED TO ALIQUOTS OF BRAIN HOMOGENATE

Endogenous serotonin	Serotonin added	Total serotonin		Recovery of total serotonin
		Calculated	Found	
μg	μg	μg	μg	per cent
1·60	0·80	2·40	2·45	102
1·72	0·80	2·52	2·47	98
1·34	0·80	2·14	2·01	94
1·92	0·80	2·72	2·67	98
1·60	0·80	2·40	2·28	95

other. Norepinephrine was determined by oxidation, at pH 5, to a highly fluorescent trihydroxyindole[2] and serotonin by its native fluorescence in 3 N hydrochloric acid.[1]

When one volume of salt-saturated 0·01 N hydrochloric acid containing serotonin is shaken with ten volumes of *n*-butanol, about 85 per cent of the serotonin is extracted into the organic phase. Serotonin added to aliquots of rabbit brain homogenates was almost completely recovered (Table 1), and the recovery of norepinephrine added to the same aliquots was the same as previously reported.[2]

The amount of serotonin found in rabbit brainstem by this technique is almost identical with that obtained by the use of the alkaline extraction procedure of Bogdanski *et al.*[1] The single extraction method yielded a mean serotonin content for normal rabbit brainstem of 0·69 μg/g ± 0·05 (s.d.), as compared with 0·66 μg/g ± 0·03 (s.d.), with the alkaline extraction procedure. The serotonin and

norepinephrine levels in the brainstems of cat and dog obtained with the single extraction method were also comparable to those found when the separate extraction procedures were used. Tryptamine, metanephrine, normetanephrine and 3,4-dihydroxybenzylamine, which are also extracted by *n*-butanol from a salt-saturated acid solution, do not interfere with the determination of norepinephrine or serotonin.

5-Hydroxytryptophan (5HTP), which has fluorescence characteristics similar to those of serotonin, is partially extracted at the acid pH level. Usually this does not create a problem because 5HTP is not present in detectable amounts in tissues. However, on administration of 5HTP to raise the tissue level of serotonin, it is advisable to analyze the brain for serotonin by the method of Bogdanski *et al.*, which incorporates a wash to remove the 5HTP.

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Inhibition of the enzymic oxidation of some dihydropyridines by polycyclic aromatic hydrocarbons

(*Received* 13 *January* 1961)

A SOLUBLE flavoprotein that catalyzes the oxidation of reduced ribosyl nicotinamide (NRH) by vitamin K_3 was recently isolated from rat kidney and seminal vesicle.[1] This enzyme also catalyzes the oxidation of certain N^1-alkyl derivatives of dihydronicotinamide, but it is completely inert toward the reduced forms of triphosphopyridine nucleotide, diphosphopyridine nucleotide, and nicotinamide mononucleotide (TPNH, DPNH and NMNH, respectively). The NRH-oxidizing enzyme can be separated from other soluble proteins which oxidize either TPNH or DPNH, or both, in the presence of vitamin K_3 and other quinones or dyes,[2, 3, 4] and does not seem to be identical with any flavoprotein previously described. We have recently observed that the enzymatic oxidation of N^1-(*n*-propyl) dihydronicotinamide is inhibited by very low concentrations ($<10^{-6}$ M) of benz(*a*)anthracene and some derivatives of this hydrocarbon, as shown in Table 1. The oxidation of NRH was also inhibited by these hydrocarbons to about the same extent. On the contrary, the oxidation of DPNH by vitamin K_3, catalyzed by a kidney enzyme with properties very similar to the flavoprotein described by Märki and Martius[5], was quite unaffected by benzo(*a*)pyrene and 7,12-dimethylbenz(*a*)anthracene at a concentration of 10^{-7} M. Experiments carried out under different degrees of illumination suggested that light was not necessary for the inhibition of the NRH-oxidizing enzyme by benzo(*a*)pyrene. Analysis of the inhibition by the latter aromatic hydrocarbon, according to the method of Lineweaver and Burk[5], showed that it was not competitive with respect to vitamin K_3.

It was also observed that those polycyclic aromatic hydrocarbons that inhibited the NRH-oxidizing enzyme formed red-colored 1:1 molecular complexes with naphthoquinones such as vitamins K_1, $K_{2(5)}$, $K_{2(10)}$ and K_3. Spectrophotometric studies of equimolar solutions (10^{-3} M in methylene chloride) of these benz(*a*)anthracene derivatives and vitamin K_3 revealed that the complex formation was accompanied by the appearance of broad, unstructured absorption bands with maxima in the

range of 480–490 mμ. The intensity of these bands was greatly enhanced by cooling to −78° C, a process which was readily reversible by warming the solutions to room temperature. Intermolecular charge transfer[6, 7] almost certainly occurs in these complexes. The relationship of the electron-donating properties of polycyclic aromatic hydrocarbons[8] to their ability to inhibit the NRH-oxidizing enzyme remains to be determined.

TABLE 1. INHIBITION OF THE ENZYMATIC OXIDATION OF N^1-(*n*-PROPYL) DIHYDRONICOTINAMIDE BY POLYCYCLIC AROMATIC HYDROCARBONS

Aromatic hydrocarbon	Percentage inhibition		
Final concentration	6×10^{-7} M	6×10^{-8} M	6×10^{-9} M
Benz(*a*)anthracene	72	38	7
7-Methylbenz(*a*)anthracene	79	61	26
7,12-Dimethylbenz(*a*)anthracene	73	46	22
Benzo(*a*)pyrene	79	51	33
3-Methylcholanthrene	32	28	9
1,2-Cyclopentenophenanthrene	0	0	0
Anthracene	0	0	0
Phenanthrene	0	0	0
Naphthalene	0	0	0
Fluorene	0	0	0

The reactions were carried out at 25° in a final volume of 3·0 ml containing 250 μmoles of tris(hydroxymethyl)amino methane buffer of pH 8·5, 0·15 μmole of vitamin K_3 (in 0·01 ml of ethanol), 0·35 μmole of N^1-(*n*-propyl)dihydronicotinamide and suitable amounts of enzyme. The polycyclic aromatic hydrocarbons were freshly dissolved in ethanol (with precautions to combat photo-oxidation), and were added to the reaction mixture in a volume of 0·01 ml (the same volume of ethanol was added to the control cuvettes). The oxidation of the dihydropyridine was followed by decrease in absorbancy at 360 mμ, and was corrected for any non-enzymatic reactions. The rates of reaction were obtained from zero-order plots of the initial rate of change in absorbancy versus time, the oxidations being initiated by the addition of enzyme. The enzyme was isolated from ultracentrifuged homogenates of rat kidney by a procedure involving (a) fractionation with ammonium sulfate (50–60 per cent saturation), (b) removal of inert protein after heating to 40° for 20 minutes at pH 4·2, (c) treatment with protamine sulfate, and (d) adsorption to and elution from calcium phosphate gel. One mg of the purified enzyme catalyzed the oxidation of 15 μmoles of N^1-(*n*-propyl)-dihydronicotinamide per min under these conditions.

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SECOND SCANDINAVIAN SUMMER MEETING OF BIOCHEMISTRY, PHARMACOLOGY AND PHYSIOLOGY

August 27–29, 1959, Turku, Finland

IN THE course of this meeting, there were papers read on the following topics: the Biochemistry of Subcellular Particles; the Biochemistry of Connective Tissues; the Mechanisms of Glucuronide and Sulphate Conjugation; and the Central Control of the Autonomous Nervous System.

On the following pages there appears a second and final selection of papers from this symposium, the first of which appeared in *Biochemical Pharmacology* Volume 5 Number 4.

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BIOCHEMISTRY OF EXPERIMENTAL LATHYRISM

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Abstract—Experimental lathyrism may be of importance in an understanding of diseases of connective tissue, since symptoms of lathyrism occur as a weakness of fibrous tissue and disordered growth of cartilage and bone. Changes observed in peripheral tissue and general metabolic disturbances are discussed. The collagen content of skin may be altered. Experimental evidence showed that the alkali-soluble hydroxyproline was increased five-fold; but good analytical data were not obtainable on the constituents of alkali-soluble collagen. The deposition of collagen was studied. The metabolic defect can be approached by analysis of blood and urine, and by study of the metabolism of β-aminopropionitrile. It is suggested that in lathyrism the general metabolism of nitrogenous components is disturbed.

FOR practical reasons lathyrism would hardly deserve much attention for its own sake. The interest is due to the similarity between its symptoms and those of many changes adherent to diseases of the connective tissue.[1] When the mechanism of experimental lathyrism is understood, it will appreciably contribute to our understanding of the formation and degeneration of the connective tissue. Also means can be developed for the prevention of undesirable fibrous scars and adhesions.

The essential features of lathyrism caused by sweet peas and specifically by their content of β-aminopropionitrile have been reviewed frequently.[2, 3] The symptoms manifest themselves as a weakness of the fibrous tissue and as a disordered growth of cartilage and bone. Also the nervous system is affected, but here I limit myself to the connective tissue. As the starting point we may recall the histological description of healing wounds in lathyritic rats.[4, 5] "The granulation tissue was thinner, and showed numerous extravasated red blood cells and foci of an eosinophilic material containing fine, disoriented fibrils. The number of fibroblasts per unit area was decreased and throughout the experimental period these cells were more spindle-shaped. The number of collagen fibrils was also decreased. In the cut edge of panniculus carnosus the fibroblastic proliferation was more intense in experimental than in control animals".[4]

All the connective tissue changes may be explained by the defect in the deposition of collagen fibres: the weakness of healing scars, the aneurysmas of aorta and hernias, the retardation in weight gain, disordered growth of cartilage cells and the malformations. I suggest that the breaking of the regular columns of cartilage cells[6] and the formation of teratomas[7] in lathyrism are related, and both are due to the lack of a guiding fibrous matrix.

The deviations from the normal in connective tissue can be discussed at two levels: (a) the changes observed in the peripheral tissue, and (b) the general metabolic disturbance.

PERIPHERAL TISSUE

Some investigators claim that the *collagen* content of the skin is not altered,[2] that its electron-microscopic appearance[1] and the thermal shrinking point[8] are regular. However, in a healing wound the number of the fibrils[4, 9] and the tensile strength[8] are decreased, as also the formation of hydroxyproline in the granuloma tissue.[10] The fragility of chick embryos is increased in lathyrism.[11] Some of the conflicting results depend on the experimental conditions, and the defect is revealed only in circumstances where the collagen fibre formation is put to a special test and not complicated by reactive fibroblastic proliferation in sites where the tissue is dislocated by the mechanical stress.

In view of these statements we wished to obtain information on the *precursor fractions of collagen*. In scorbut or adrenocortical hyperfunction the precursor of the fibrous collagen, the alkali- or neutral salt-soluble collagen, is decreased indicating that the defect in the fibre formation lies, at least partly, in protein synthesis.[12, 13] Quite the contrary seems to be the case in lathyrism. The alkali-soluble hydroxyproline is increased fivefold.[14] This is in agreement with those authors[15] who have shown that in lathyrism there is a defect in the precipitation of the collagen fibres in the cartilage but that the total content of collagen is not changed. Other recent reports also indicate an increased solubility of collagen in lathyritic rats[5] and chick embryos.[11]

As yet we have not obtained qualitative data on the alkali-soluble collagen in lathyrism, but it is observed that its precipitation *in vitro* during dialysis or storage is appreciably retarded for non-explained reasons. The proportion of the "directly reacting" (without hydrolysis) hydroxyproline was also clearly lower in lathyrism. We have observed an increase in the apparent tyrosine content of the gelatine derived from lathyritic bones,[8] but the possibility of impurities in the gelatine is not excluded and may be due to the relatively increased amount of blood vessels. Levene and Gross[11] found that the specific optical rotation, intrinsic viscosity and sedimentation constant of the collagen extracted from the treated chick embryos were essentially normal. They also concluded that the soluble collagen originates from fibrils formed prior to treatment with lathyrogenic agent. The extracted collagen could be reconstituted to typical striated fibrils.[11] Its molecular weight and axial ratio are normal.[22] Electron microscope analysis[16] shows that extraction of lathyritic tissue with cold 1 M saline causes a dissolution of the fibrils to fine filaments.

The available knowledge on the composition of other elements in the connective tissue is not quite unanimous. The incorporation of radioactive sulphate is increased in the femurs of the rats,[17] but this can be interpreted rather as a reaction similar the fibroblastic proliferation at the edges of the wound. We have some of our own data[14] indicating that the salt-soluble uronic acid was increased, especially when determined with carbazole reaction, indicating in the first place glucuronic acid and hence chondroitin sulphates A and/or C. Also it has been claimed that mucopolysaccharides are increased in the aorta,[18] but not in the skin. This is in some disagreement with the finding that galactosamine is decreased in growing epiphysial cartilage.[19, 20] This is confirmed but the mechanism is obscure.[21] Also it is worth mentioning that the neutral salt-soluble non-collagenous protein of the skin is increased.[14, 15]

METABOLISM

In studies of the peripheral tissue we have a concrete approach, i.e. the deposition collagen. The inability of the soluble collagen molecules to form stabilizing linkages

must have its original cause in the intermediary metabolism. Two lines of research are obvious to begin with: (a) the routine analyses on blood and urine, and (b) the metabolism of the β-aminopropionitrile itself.

The analyses of blood and urine have not contributed very much. Blood sugar and non-protein nitrogen are within normal limits;[22] on blood calcium, phosphorus and phosphatase the reports are so conflicting that no clear picture emerges.[3] It may be concluded that the mineral metabolism is not primarily affected. The renal readsorption of some substances (including tyrosine and histidine) is impaired.[23] The injurious substance, β-aminopropionitrile, has been detected in the urine[24] as also its detoxication product, cyanoacetic acid.[25] The stimulated formation of the red cells and haemoglobin[26] indicates an increased need of oxygen, and is discussed below.

Considering the occurrence of cyanoacetic acid in urine and the fact that the amino group must not be substituted but the cyano group can be replaced by SH group, it may be concluded that the amino group is the essential radical and that the detoxication involves a deamination. This approach was developed further by studying the effect of the inhibition of monoamino-oxidase (by iproniazid) on the development of lathyrism,[28, 28a] which was found to be aggravated. Corroborating evidence was sought with other hydrazines which do not inhibit the monoamino-oxidase. *Iso*niazid also has a similar effect on lathyrism[22] but is a weak inhibitor of monoamino-oxidase.[29] Carbazides and other hydrazines alone have been reported to cause symptoms in bone[30] similar to those caused by β-aminopropionitrile, and other mechanisms for the effect of iproniazid should not be overlooked in addition to the inhibition of monoamino-oxidase.

The deamination requires oxygen and it can be shown that in the presence of β-aminopropionitrile the oxygen consumption of the liver slices and epiphysial cartilage is increased by 50 per cent[27] which is in keeping with the increased hematopoiesis in the intact animal. However, the increase exceeds by far the calculated requirement for the detoxication of all the β-aminopropionitrile, and about one-tenth of the β-aminopropionitrile disappears. In liver slices from lathyritic rats the oxygen consumption was markedly decreased but again stimulated by β-aminopropionitrile. On dehydrogenation no change was observed (studied with Thunberg-technique with various substrates)[22].

Since these findings implicate the energy metabolism, the content on energy-rich nucelotide phosphates was determined in the muscles and in chick embryos. In chick embryos the nucleotides did not change in any consistent manner.[31] In muscle there are differences in the nucleotide phosphates, but only at the later stages, and they may be due to secondary reasons.[22]

When the experimental diets were supplemented with sucrose, the signs of osteolathyrism were not very much affected, but the growth was definitely delayed in animals on the lathyritic diets.[22] On its face value, this result means that in lathyrism the utilization of sucrose is impaired and this is indicated by the retardation of the growth. All this presumes that the growth retardation can be an independent sign of lathyrism.

Many feeding experiments[2, 3] have been carried out to elucidate the mechanism of lathyrism. The working hypothesis has been that β-aminopropionitrile causes an unknown deficiency of some metabolic factors, and this deficiency would be compensated by suitable additions in the diet. This belief has a sound basis. In animals on

good diets the lathyrism develops slowly and can and be almost prevented when the diet is supplemented with protein. Several claims have been staked on the values of individual amino acids but the evidence has always remained in conclusive.[3] Neither has it been possible to find defects in the metabolism of individual amino acids or in their concentrations in the cartilage,[20] or chick embryo,[31, 32] except a reversal in the ratios of proline and hydroxyproline.[32]

The significance of proteins in the feeding experiments and the essential role of the amino group in the β-aminopropionitrile suggest the importance of studying *nitrogen metabolism*. Two lines seemed especially attractive, glutamine metabolism and transamination. Since the β-aminopropionitrile appears in the peas as an amide-like conjugate with glutamic acid, it was necessary to discover whether it interferes in the synthesis of glutamine. However, thus far all our attempts with brain glutamine synthetase have been negative or inconclusive. The experiments with heart transaminase were also quite negative. It is still too early, though, to conclude that these functions were not disturbed *in vivo*, where numerous other reactions and energy metabolism contribute to the final result.[33]

We have also some data[22] on the synthesis of nitrogenous components from ammonia marked with ^{15}N. In chick embryos the incorporation of ammonia nitrogen into purines was, after 4 days, exactly the same in normal and β-aminopropionitrile-treated eggs which otherwise showed good evidence of lathyrism. This seems to eliminate the nuclear metabolism from further consideration. Nor was there any difference in the incorporation of ammonia nitrogen into lipids or into trichloroacetic acid-soluble fraction (measured 3 days after injection of ammonium chloride into the yolk sac).

In chick embryos the incorporation of the β-nitrogen of β-aminopropionitrile was so low that the nitrile could not be used in concentrations high enough. Its detoxication by deamination was already discussed. It is rapidly removed from the body.[25] Some data are known on the metabolism of aminoacetonitrile, which is equally toxic, but its own metabolism is obviously different.[34]

CONCLUDING COMMENTS

In lathyrism the fibrous collagen is decreased, in spite of the abundance of its precursor. Secondary proliferation of the connective tissue is common, where the mechanical stress dislocates the originally weakened tissue.

The β-amino group is an essential radical of the aminopropionitrile. The symptoms of lathyrism are aggravated by hydrazines, including a monoamino-oxidase inhibitor, iproniazid. The detoxication involves an oxidative deamination, which partly explains the increased oxygen consumption by the tissue slices in the presence of β-aminopropionitrile and the stimulated haematopoiesis *in vivo*.

Brain glutamine synthetase or heart transaminase were not inhibited *in vitro* by β-aminopropionitrile. The nuclear metabolism does not appear to be disturbed.

It is suggested that in lathyrism the general metabolism of nitrogenous components is disturbed.

Acknowledgement—Our work presented above was supported by Sigrid Jusélius Foundation and the State Board of Science.

Note added in proof—The incorporation of $^{15}NH_4Cl$ into the collagen fractions in chick embryos was higher in lathyritic embryos than controls 2–3 days after the

simultaneous injection of β-aminopropionitrile and $^{15}NH_4Cl$. When the β-aminopropionitrile was administered 4 days later than $^{15}NH_4Cl$, it caused the replacement of the labelled nitrogen by non-labelled. It is not known whether the synthesis of the individual amino acids was affected, but there seems to be no disturbance in the conversion of proline to hydroxyproline.[35]

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BIOCHEMICAL CONJUGATION PROCESSES

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Abstract—Biochemical processes of conjugation include the formation of glucuronides, sulphuric esters, sulphamates, phosphoric esters and mercapturic acid derivatives. A result of the conjugation is the production of compounds more ionized and more water soluble than parent compounds. The significance of these substances in the animal kingdom is pointed out. Mercapturic acid formation from naphthalene as well as the general significance of conjugation processes in detoxication are discussed.

KNOWLEDGE and understanding of the biochemical processes of conjugation have greatly increased during the past decade. These biochemical processes which include the formation of glucuronides, sulphuric esters, sulphamates, phosphoric esters and mercapturic acid derivatives generally lead to the production of compounds which are more water soluble and more readily ionized than the original substances. The recent discovery that 2-naphthylamine is excreted as bis (2-amino-1-naphthyl) phosphate suggests that many biochemical reactions of this type may still be discovered.

Brodie and his colleagues[1, 2] have shown how the ionized compounds are more readily excreted by the kidney. The ability to form these derivatives which are easily excreted is essential to animals living on land, and work from Brodie's laboratory shows that fish and aquatic amphibia do not have the ability to form glucuronides. The fact that mammalian foetal tissues, and new-born animals do not possess the ability to form the conjugates is in agreement with the idea that the development of these systems for the metabolism of unionized compounds is associated with, and probably essential for, terrestrial life.[3] The vertebrate animals which do not form glucuronides appear to lack the ability to oxidize uridine diphosphoglucose to uridine diphosphoglucuronic acid or "active" glucuronide.

The work of Smith[4] on the metabolism of foreign compounds by insects is of interest in this respect. This shows that these terrestrial animals convert phenols and other related compounds to glucosides instead of the glucuronides formed by mammals. This is an example of biochemical evolution in which the problem of maintaining the internal environment has been attempted in a different way.

The ionization theory which Brodie has developed extends earlier work such as that of Quick[5]. There are difficulties in the theory. The metabolic processes of conjugation produce ionized derivatives which cannot penetrate cell walls and so are not reabsorbed by the kidney. The reactions leading to products which cannot penetrate the cell membrane proceed inside the cell. In this case it must be difficult for these products to diffuse out of the cell so that they should accumulate within the cells.

The early work on glucuronides, which was mainly descriptive, showed that phenols, alcohols and carboxylic acids are excreted as glucosiduronic acids, but more recent investigations have revealed the occurrence of glucuronides of primary aromatic

amines[6] and in one case of a thiol (mercapto benzthiazol).[7] In view of these findings it might be of interest to see if aliphatic amines and secondary amines are excreted as N-glucuronides and if organic selenium derivatives are excreted in an analagous way to the mercapto benzthiazol.

Until a few years ago sulphate conjugation was only known to occur with phenols, but it is now known that aromatic amines are excreted as N-sulphates or sulphamates[6] and Roy[8] has shown that such compounds are formed *in vitro* when active sulphate is present. More recently Vestermark and Boström[9] have shown that simple alcohols and glycols are also excreted as ethereal sulphates.

The most remarkable of the new conjugates, however, is the bis (2-amino-1-naphthyl) phosphate found by Troll *et al.*[10] as a metabolite of 2-naphthylamine in dog urine. This derivative has two aromatic residues to each phosphate group and is unstable in solution. Dr. Manson has synthesized the derivative by reduction of the corresponding nitro compound and the pure product has now been isolated and has chromatographic properties identical with a substance sometimes present in the urine of dogs treated with 2-naphthylamine.

We have been interested in mercapturic acid formation from hydrocarbons such as naphthalene. When *p*-bromophenyl mercapturic acid was isolated by Baumann and Preusse,[11] these authors were aware that the material was excreted in the urine in some form which was changed on acidification, because there was a large irreversible change in optical activity on acidification of the urine. Although the precursor of *p*-bromophenyl mercapturic acid has not been isolated, the precursor of naphthyl mercapturic acid has been isolated as the ammonium salt in my laboratory.[12] The precursor is decomposed by cold acid or by cold alkali, but is stable enough to be crystallized as the ammonium salt. It is 1 : 2-dihydro-2-hydroxy-1-naphthyl-S-acetylcysteine which readily eliminates water to give the 1-naphthyl mercapturic acid, first isolated by Bourne and Young[13].

Studies of the intermediate reactions in the biosynthesis of the naphthalene mercapturic acid precursor by rat tissue homogenates[14] have shown that the first stage appears to be the formation of 1 : 2-dihydro-2-hydroxy-1-naphthyl-S-glutathione by liver tissue. This is then broken down to the corresponding cysteine derivative by kidney enzymes. The dihydrohydroxynaphthyl cysteine is then acetylated in the liver to give the mercapturic acid precursor. These steps must occur rapidly in the animal as we have detected the naphthyl mercapturic acid precursor in the urine of rats within 20 min of administration of naphthalene.

The naphthalene derivative which reacts with glutathione could be the hypothetical 1 : 2-epoxide which we have been unable to synthesize chemically. We have, however, administered 1 : 2-dihydro-3 : 4-epoxynaphthalene to rats and this is excreted as the corresponding mercapturic acid precursor. In this case the corresponding tetrahydro-hydroxynaphthylcysteine (i.e. not acetylated) is also excreted and has been isolated.

These experiments support the hypothesis that the microsomes convert naphthalene and other hydrocarbons into epoxides or similar derivatives which can then react either with water to yield dihydrodiols or with glutathione to yield eventually mercapturic acid precursors.

Dr. Manson has evidence that 2-naphthylamine is excreted both as the 1-mercapturic acid and as a precursor of a 5- or 6-mercapturic acid. These derivatives are, however, difficult to isolate or to synthesize.

The conjugation reactions with sulphate or with glucuronide may be used as transporting mechanism. If a hormone inactivated by conjugation is carried by the blood stream to an organ or tissue rich in β-glucuronidase the active hormone could be released locally and produce its particular biological effect. Although such a mechanism is possible there is no proof that it actually occurs. To attribute a purpose to biochemical processes is difficult in this as in other cases.

Although conjugation processes usually lead to detoxication there is a possibility that the conjugation of the *o*-aminophenols formed by metabolism of carcinogenic aromatic amines changes the site of action of the parent amine. Men exposed to 2-naphthylamine and dogs treated with 2-naphthylamine develop cancer of the bladder, and cancer of the bladder only. We think that the parent amine is not carcinogenic but that one of the first formed metabolites, 2-amino-1-naphthol, is carcinogenic locally. When 2-naphthylamine is adsorbed it is metabolized mainly in the liver to 2-amino-1-naphthol. In the liver it is immediately conjugated with glucuronide, sulphate and presumably phosphate. These conjugates are then excreted and concentrated in the kidney. In the urine the glucuronide or the phosphate can be hydrolysed enzymatically to give the free carcinogenic 2-amino-1-naphthol. This mechanism would explain the localized action of aromatic amines such as 2-naphthylamine on the bladder. The corresponding sulphate is not hydrolysed by urinary sulphatase but it might be hydrolysed by some other mechanism.

Levvy[15] showed that the hydrolysis of glucuronides by β-glucuronidase is inhibited by 1 → 4 saccharolactone much more strongly than by 3 → 6 saccharolactone. Investigation of this inhibition at different pH values has shown that the inhibition is much less at pH values above the optimum.[16] The pH–enzyme activity curve and the pH–inhibition curve are parallel, which agrees with the idea that the substrate and inhibitor combine with the same centre on the enzyme. The 1 → 4 saccharolactone has only a fraction of the inhibitory effect at pH 7 compared with its effect at the optimu pH for the enzyme pH 5.

Because the so-called 1 → 4 saccharolactone is such an effective competitive inhibitor of β-glucuronidase one would expect the saccharolactone and glucuronide molecules to have similar structures. The glucuronides have, however, pyranose structures, while the so-called 1 → 4 saccharolactone would have a furanose ring. Dr. Lumley Jones has found that the carbonyl stretching band of the so-called ammonium 1 → 4 saccharolactone is at 1744 cm^{-1} which would be in agreement with a pyranose structure. The potassium salt of 3 → 6 saccharolactone has a band at 1780 cm^{-1}, in agreement with a furanose ring structure. On the other hand the lactone at present designated as the 1 → 4 form reacts very slowly with one molecule of periodate, in agreement with the furanose structure.

One difficulty in the study of conjugation processes is the variation in the reaction. For example many workers have observed the production of free naphthalene on acidification of the urine of rats dosed with naphthalene. Ten years ago "naphthalene urine" always contained the naphthalene precursor. Our rats and rabbits treated with naphthalene now do not excrete the naphthalene precursor. In the past few years we have administered 2-naphthylamine to many animals. In the first experiments in which ^{14}C labelled 2-naphthylamine was injected into dogs, 2-amino-l-napthyl sulphate was the only metabolite detected in the urine. In later experiments under the same conditions 2-amino-1-naphthyl glucosiduronic acid, 2-amino-1-naphthyl sulphate,

the phosphoric ester and other products were excreted. These examples of apparent variation indicate that there are many factors concerned in the metabolism of foreign compounds which are not yet understood.

In the study of conjugation reactions, there are probably new types of reactions still to be discovered, although the main part of the descriptive phase is probably complete. Much remains to be investigated in the conjugation of products of normal metabolism as compared with drugs and other foreign compounds. We know a great deal about the mechanism of the reactions and we appear to understand their function or purpose better than we did. It still remains, however, an interesting field for research.

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THE MECHANISM OF GLUCURONIDE FORMATION

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Abstract—The mechanism of formation of simple glucuronides in animal tissues is reviewed. So far glucuronide synthesis has been found to occur in liver and to a lesser extent in kidney of a number of animal species. Of the mammals studied cat liver slices formed very little glucuronide. More recently glucuronide formation has been discovered to occur in slices of gastrointestinal mucosa supplied with glucose. It would appear that a necessary requisite for the formation of glucuronides is the presence of glycogen or feeding of animals with carbohydrates which points to the essentiality of carbohydrates in the mechanism of synthesis. Starting with glucose the possible steps in glucuronide metabolism are given.

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THIS short review will be restricted to the mechanism of formation of simple glucuronides within the animal body. It will deal with the elucidation of this mechanism and with the various tissues in which it is found. No attempt will be made to present a complete picture of glucuronide metabolism and its significance, which would be impossible in this compass.

STRUCTURE OF GLUCURONIDES

Glucuronic acid may be regarded as a D-glucose molecule in which the alcoholic group at carbon atom (6) has been oxidized to that of a carboxylic acid. The reducing properties at carbon atom (1) still remain, however, and the molecule is therefore still able to form glycosides, termed in this case glucuronides or, more accurately, glucosiduronic acids. These glucuronides are of two main kinds: the "ether" type in which carbon atom (1) of glucuronic acid is joined to an hydroxyl group of the aglycone by a relatively stable glycosidic bond, and the "ester" type, in which this carbon atom is linked with a carboxyl group of the aglycone through the much less stable acylal bond. The aglycones can be aliphatic, alicyclic, aromatic or heterocyclic. Linkage may also in a few cases be through a nitrogen or sulphur atom, with an amine or a mercapto compound, respectively; the former is extremely unstable,[1] and the latter has not yet been much investigated.[2] (For comprehensive treatment of the chemistry and occurrence of glucuronides, see Teague[3].)

HISTORICAL

The first glucuronide was unwittingly isolated in 1855 by Schmid[4]; Jaffe[5] and Schmiedeberg and Meyer[6] were the earliest to identify glucuronic acid in such a conjugate. At this time, hypotheses on glucuronide formation necessarily lacked experimental backing. Schmiedeberg and Meyer,[6] for example, suggested that glucose was normally oxidized through glucuronic acid in the course of its complete bodily

oxidation to CO_2 and water; any aglycone that happened to be available was captured by this glucuronic acid and a glucuronide thereby formed. It was difficult to see how carbon atom (6) of simple glucose could be oxidized without the more reactive carbon atom (1) undergoing the same fate, and other workers[7, 8] postulated that glucose itself first combined with the aglycone and that the resulting glucoside, its carbon atom (1) masked, was then oxidized to the glucuronide. When evidence became available, this was shown to be unlikely, for it seemed that glucosides were rapidly hydrolysed in the body, no more glucuronide appearing after their administration than if the free aglycone had been given, and in both cases formation also of the aglycone sulphate was observed.[9] (For an excellent historical review, see Williams[10].)

EVIDENCE FOR MECHANISM

One pathway of glucuronide synthesis is now known, which savours a little of each of these early hypotheses. Its elucidation will now be described.

The first clues were obtained with work on whole animals. It was soon apparent that liver was a major site, and that this organ formed glucuronides more efficiently if it contained glycogen, or if the animals were fed carbohydrate.[11, 12] Though argument raged hot over possible precursors of glucuronic acid, it was obvious that, beyond illustrating such indubitable connexions between glucuronide formation and carbohydrate availability, results with intact animals were inadequate.

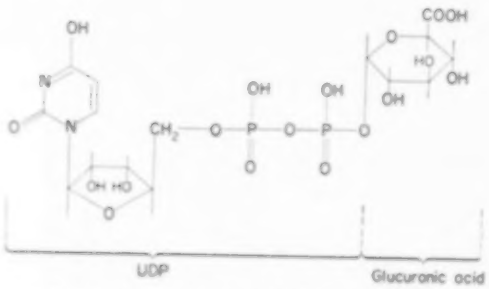

FIG. 1. Uridine diphosphate glucuronic acid.

Considerable advances came with the advent of techniques for isolated tissues. Lipschitz and Bueding[13] and Storey,[14] using slices from these organs, showed that glucuronide synthesis occurred in liver and, to less extent, in kidney. Various aglycones were employed, and for them all the process appeared strictly aerobic, most probably involving endergonic phosphorylative reactions.

About the same time evidence from work with isotopes was becoming available, and although this was to some extent conflicting it did make clear that the carbon chain of administered glucose came down unbroken to the glucuronic acid of excreted glucuronide.[15–17] Glucurone or glucuronate was not a direct source.[18]

To pursue the question further, broken cell preparations were employed.[19] The rapid and reasonably specific method of Levvy and Storey[20] was modified to study formation of *o*-aminophenyl glucuronide in liver homogenates, and it was soon clear that an unknown factor was involved. Synthesis did not occur unless a boiled liver extract

was added, and no known compound, even the likely glucuronic acid-1-phosphates, could reproduce this effect. The unknown factor was isolated, and on analysis uridine, stable phosphate, labile phosphate and glucuronic acid were found in equivalent proportions;[21] the parent "uridine diphosphate glucuronic acid" (UDPglucuronic acid) was analagous to the recently discovered UDPglucose.[22] The nucleotide was in effect an "active form" of glucuronic acid and transferred this molecule to the aglycone under the influence of an enzyme (preferably termed "UDPtransglucuronylase": see Dixon and Webb[23]). The transfer was anaerobic and seemed to represent the final stage in that formation of glucuronides observed in liver slices.

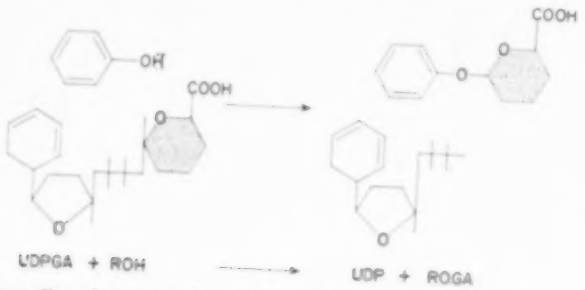

FIG. 2. Schematic outline of glucuronyl transfer from UDPglucuronic acid to an acceptor substrate

The preceding stages presumably formed UDPglucuronic acid itself, and were soon clarified. Strominger *et al.*[24] showed that UDPglucuronic acid was immediately derived from the oxidation of UDPglucose, diphosphopyridine nucleotide (DPN) and oxygen was required together with the enzyme UDPglucose dehydrogenase. UDP-glucose can be made by the action of a pyrophosphorylase on uridine triphosphate (UTP) and α-glucose-1-phosphate; UTP is of course dependent on the supply of adenosine triphosphate (ATP). The constituent reactions may therefore be outlined as follows:

$$\text{Glycogen} \xrightarrow{H_3PO_4} \alpha\text{-glucose-1-phosphate} \quad (1)$$

$$\alpha\text{-Glucose-1-phosphate} + \text{UTP} \rightarrow \text{UDPglucose} + \text{PP}^{25} \quad (2)$$

$$\text{UDPglucose} \xrightarrow{\text{DPN},\ O_2} \text{UDPglucuronic acid}^{24} \quad (3)$$

$$\text{UDPglucuronic acid} + \text{R.OH} \rightarrow \text{R.O.glucuronic acid} + \text{UDP}^{19,\ 21,\ 26-28} \quad (4)$$

Production of glucuronides by this pathway touches on both the early suggestions quoted: a line of glucose oxidation is followed, but carbon atom (1) is masked by UDP. Observations that carbohydrate, oxygen and energy are required, that the glucose chain comes down unbroken and that free glucuronic acid plays no part are all now explicable by this scheme.

It is also evident that no selective "chemical defence" is operating (as was once supposed) with conjugation only of ingested toxic compounds, for enzymic transference of glucuronic acid from UDPglucuronic acid occurs for all aglycones (except sugars) so far examined: for ether and ester types, aromatic or aliphatic, "foreign" or

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"physiological",[25] and for aromatic amines;[1] thyroxine,[27] various steroids[27, 28] and bilirubin[29] are examples of particular physiological aglycones handled in this way. Whether the same enzyme transfers glucuronic acid to all these substrates is so far uncertain, but in each case it is located in the microsomes, is unstable, requires Mg ions and has a fairly broad pH optimum around pH 7·6.

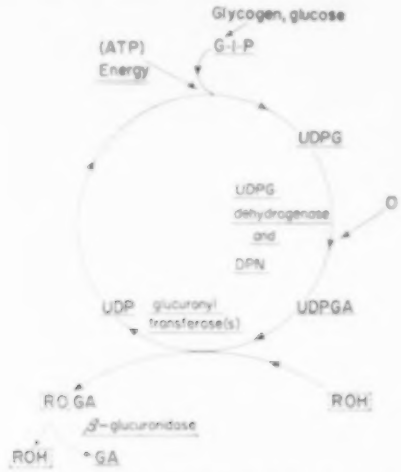

FIG. 3. Simplified scheme of glucuronide formation. G-1-P, glucose-1-phosphate; UDP, uridine diphosphate; G, glucose; GA, glucuronic acid; ROH, acceptor substrate. UDPtransglucuronylase is here termed, less satisfactorily, "glucuronyl transferase".

OCCURRENCE OF THIS HEPATIC MECHANISM

The glucuronyl transfer described above occurs in liver of man, pig, sheep, dog, rabbit, guinea pig, rat, mouse, pigeon, hen and frog,[30, 31] a sex difference being reported in the rat.[32] The system may be present to a very limited extent in brown trout liver, being there extremely thermolabile,[32] but is apparently absent from other fish and from tadpoles.[33] Among mammals, the cat is an exception. Hartiala[34] pointed out that cat liver slices form very little glucuronide, and this has been confirmed for the intact animal by others;[35, 36] the reason is not lack of UDPglucuronic acid, but of UDPtransglucuronylase.[30]

Conjugation with glucuronic acid is also low in foetal and neonatal liver,[37, 38] where it increases with growth. Since bilirubin is excreted largely as a glucuronide[39–41] and excess unconjugated bilirubin is typical of non-haemolytic non-obstructive "physiological" jaundice of the new-born, foetal conjugating mechanisms are of interest. Work with livers of infant mice and foetal guinea pigs [30, 42] demonstrated the low conjugation to be due, not to hydrolysis of formed glucuronide by the large amount of β-glucuronidase present in infant tissues or to an inhibitory factor in foetal liver, but to true low levels of both UDPglucuronic acid and UDPtransglucuronylase; these were virtually absent in new-born mice, in foetal guinea pig up to 5 weeks and in the 3–4 month human foetus. The guinea pig does not reach adult levels in these substances until several days after birth. This has been shown for several aglycones, including bilirubin.[43, 44] It might therefore be that "physiological" jaundice of the new-born is due to survival of foetal characteristics into the neonatal state.

Several other jaundiced conditions, in both rat and man, are accompanied by reduced conjugation of bilirubin and other aglycones with glucuronic acid; in these cases only the UDPtransglucuronylase may be deficient.[45–49]

Bilirubin can be conjugated also with sulphate[50] and one might expect this pathway to be followed in such animals as the cat whose glucuronide-synthesizing capacity is poor. Yet bilirubin glucuronide has been found in cat bile[51] and Lathe and Walker[43] report formation of this compound by cat liver preparations; this may be the first indication that more than one "UDPtransglucuronylase" exists in the microsomes, though evidence is by no means conclusive.

Similarly, no conclusions can yet be drawn on the induction of liver glucuronide synthesis, but in the interesting work already quoted[52] sex difference was reversible with hormone treatment and increase of UDPtransglucuronylase activity (in common with that of other microsomal enzymes) was demonstrated in new-born rats after administration of benzpyrene.

OCCURRENCE OF THIS MECHANISM EXTRAHEPATICALLY

Kidney has long been known as a site of glucuronide formation. This organ contains UDPglucuronic acid, can form it by a DPN- and oxygen-dependent enzymic oxidation of UDPglucose, and, in its cortex "microsomes", possesses an enzyme able to transfer glucuronic acid from the nucleotide to "ether" and "ester" acceptors in a manner not yet distinguishable from that of liver UDPtransglucuronylase.[53] Kidney not only resembles liver in one major pathway of its considerable glucuronide synthesis, but also in the gradual development of this pathway through foetal and neonatal life.[42]

Another important extrahepatic site of glucuronide formation has recently been discovered. Conjugation of glucuronic acid with various aglycones including steroids has been demonstrated in slices of gastrointestinal mucosa supplied with glucose.[34,54–57] This tissue, similarly, contains UDPglucuronic acid and a DPN-dependent oxidative enzyme system forming it from UDPglucose, and can transfer the nucleotide's glucuronic acid to various acceptors with the aid of an enzyme found in the particulate fraction of a water homogenate.[53] The enzyme is difficult to characterize, but resembles the UDPtransglucuronylase(s) of liver and kidney. However, in gastrointestinal tract, particularly the stomach, synthesis of glucuronides can occur by this pathway even in the early (5 week) guinea pig foetus, and is then already at, or even above, adult level.[42, 53] This level is, on wet or dry weight basis, only some 10–20 per cent that of adult liver, but must be significant in the otherwise poorly-equipped foetus. Further discussion is not relevant to this brief survey, but it should be noted that foetal tissues contain glucuronide[58] and that placenta appears to form negligible amounts.[38, 42]

Results from other sites, such as connective tissue, are not sufficiently advanced to warrant inclusion here.

OCCURRENCE OF OTHER MECHANISMS

Though the method outlined above is an important one it need not be unique. So far, there seem two possible "alternative pathways". The first is really another means

of forming UDPglucuronic acid and involves pyrophosphorolysis of UTP in the presence of glucuronic acid 1-phosphate:

$$\text{Glucuronic acid-1-P} + \text{UTP} \rightarrow \text{UDPglucuronic acid} + \text{PP}$$

This is reported in mung beans,[59] and if applicable to animals could explain the results[60] wherein ATP, UTP and glucuronate or glucurone appear (to some extent) to replace UDPglucuronic acid.

The second concerns the synthetic, or "transferase", capacity of β-glucuronidase which, it is now clear, is a completely different enzyme from UDPtransglucuronylase. Fishman and Green[61] have succeeded in demonstrating that, in β-glucuronidase preparations under special conditions of substrate concentration, transference of glucuronic acid from a glucuronide to an accepting aglycone can occur. To what extent, if any, this takes place *in vivo* is not yet clear.

This short review has necessarily confined itself to the mechanism of synthesis of simple glucuronides in animals, and many interesting digressions could not be entertained. It is well, however, to include a final note on characterization of glucuronides formed. The amounts of conjugate available in enzyme experiments do not usually permit classical analysis. It is therefore desirable to employ several substrates, the various methods of estimation or identification of whose conjugates cover not only the aglycone but also the uronic acid; this is very necessary when impure UDPglucuronic acid is being used. Hydrolysis by β-glucuronidase should be checked by its specific inhibition by saccharolactone,[62] with due regard for the substrates attacked by the enzyme.[63] Bilirubin is a peculiarly difficult aglycone and though, for example, formation of "direct" van den Bergh-reacting pigment from "indirect" has been observed in gastric mucosal preparations,[31] this by itself reflects solubility in water and not necessarily conjugate formation, still less that of a glucuronide.

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SOME ASPECTS ON SULPHATE CONJUGATION

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Abstract—Knowledge of sulphate conjugation has increased in the past decade by use of chromatography, paper electrophoresis and radioactive sulphur. Enzymic systems which are reviewed in this paper have contributed significantly to an understanding of mechanisms of sulphate conjugation. Apart from a review of enzymatic processes evidence of recent experiments is also given. In a first step step catalysed by ATP-sulphurylase, adenosine monophosphatesulphate (APS) and pyrophosphate are formed from sulphate and ATP. Catalysed by APS-kinase active sulphate (PAPS) and ADP are formed from APS and ATP. These results show the first phase in sulphate conjugation while the second phase involves transfer of the sulphate group from the activated sulphate to various compounds. This latter reaction is less understood than the more completely characterized first phase. It is important to know the nature of compounds which are conjugated with sulphate in mammalian tissues, but of considerable interest is knowledge about the nature of ester sulphates which under normal or pathological conditions occur in tissues or body fluids. Using autoradiograms, experiments are described for obtaining new data on possible sulphate receptors and on the ester sulphate patterns of body fluids.

THE credit for the discovery of conjugated sulphates and sulphate conjugation *in vivo* is usually given to Baumann on the basis of two papers published in 1876.[1, 2] At that time, Baumann was working in Hoppe-Seyler's laboratory in Strassbourg on the indigo-forming substances in urine. He observed that these compounds were decomposed by glacial acetic acid or hydrochloric acid to give some amount of sulphuric acid. He called these compounds *geparte Schwefelsäuren*, and showed that they were conjugates of sulphuric acid and organic compounds, such as phenol, the indigo-forming substance of urine, and probably several other uncharacterized compounds as well.

Another observation reported by Baumann in his first two papers was the demonstration of a raised excretion of phenyl sulphate in patients treated with carbolic acid, which at that time was a much-used antiseptic in surgical wards. He also found a marked increase in the conjugated sulphates in the urine of a dog given a dose of catechol.

In similar experiments, Baumann and Herter[3] estimated more systematically the ester sulphate excretion in various species after administration of different phenols, substituted phenols and aromatic hydrocarbons. They found that all phenols tested, and some of their substituted derivatives, increased the ester sulphate portion of the urine. A conjugation between suiphate and the compound given was proved in some cases by actual isolation of the conjugates. Some of the non-phenolic compounds tested, e.g. benzene, indol and aniline, also produced an increased ester sulphate excretion. In this case, an oxidation to the corresponding phenols was believed to

precede sulphate conjugation, a sequence of reactions later shown to be of great importance in the detoxication of various metabolites and chemicals.

Already in the classical papers by Baumann and his contemporaries which have been reviewed by several authors, among them Folin and Denis[4], Dubin[5] and Williams[6], many biochemical problems were discussed and elucidated which are still at the centre of interest in the field of sulphate conjugation.

During the past decade, the work on these problems has been facilitated and simplified to some extent by the availability of new tools in research, e.g. chromatography in its various forms, paper electrophoresis and radioactive sulphur. Important progress has also been made in recent years, particularly with respect to the enzymatic mechanisms concerned.

It is our intention to review some current trends in this work. We will also give a brief report of some recent personal investigations, which may be of certain interest in this connexion.

The opinion expressed by Baumann[1, 2] that the sulphate group of sulphate conjugates arises *in vivo* as a result of a union of sulphuric acid with the phenolic compound concerned has been a matter of much disagreement in the past. Several later workers[7, 8] were unable to confirm Baumann's findings on which he based his theory, namely, that in dogs exogenous inorganic sulphate stimulated sulphate conjugation.

Another possibility was therefore discussed, for instance by Sherwin[9] and coworkers. According to these workers, the formation of ester sulphates takes place by the oxidation in the body of mercapturic acids. Hele[10] and coworkers were, however, unable to demonstrate increased excretion of ester sulphates after the administration of mercapturic acid. On the basis of these and other studies, Hele[10] accepted Baumann's opinion of inorganic sulphate as the precursor in sulphate conjugation.

This opinion has now been strongly supported by more recent *in vivo* studies with ^{35}S-sulphate. After administration of radioactive sulphate to rats by the intraperitoneal route, Laidlaw and Young[11] in 1948 and Dziewiatkowski[12] in 1949 were able to demonstrate a high isotope labelling of the conjugated sulphate fraction of urine. A further increase in radio-sulphur in this fraction was obtained by administration of naphthylamine or phenol, simultaneously with the radioactive sulphate.

Further support for this view has been provided by the *in vitro* studies in the field of sulphate conjugation which recently culminated in clarification of the sulphate activation mechanisms.

Most of the *in vitro* studies in this field have been done with mammalian liver preparations. In 1902, Embden and Glaessner[13] were able to demonstrate synthesis of ester sulphates from phenol in perfused liver.

Experiments with liver slices in the early nineteen forties by de Meio and Arnolt[14] showed that sulphate conjugation occurred in these systems, and that thermolabile enzyme systems were involved in these processes.

Later on, as reported in 1950 and 1952, de Meio and Tkacz[15, 16] were able to demonstrate sulphate conjugation in liver homogenates. The presence in the medium of adenylic acid and α-ketoglutaric acid was required for conjugation. They also found that the sediment and supernatant of the homogenate were individually inactive. Recombination restored the activity, but not if the supernatant was previously boiled.

Their studies thus seemed to indicate that phosphorylation might be involved in the process.

Continued work by Bernstein and McGilvery[17] in 1952, and by de Meio *et al.*[18] in 1953, showed that sulphate conjugation also took place in particle-free supernatants under anaerobic conditions, if the medium contained a certain concentration of ATP and Mg ions. Moreover, Bernstein and McGilvery traced the formation of an unknown metabolite capable of stimulating sulphate conjugation, when enzyme preparations, ATP and sulphate were preincubated before the addition of phenol. Preincubation with phenol in the same system before the addition of sulphate did not, on the contrary, have this effect. These experiments seemed to indicate that two phases were involved in sulphate conjugation, the first being an activation of sulphate, and the second a transfer of sulphate to phenol.[17] Further evidence in support of the view was given by the fact that de Meio *et al.*[19] in 1955 were able to separate one enzyme called the "activating enzyme" from another one denoted as "the transferring enzyme".

Starting from the level of knowledge reached by the pioneer work just quoted, Lipmann and coworkers extended their general interest in enzymatic group activation and transfer to the field of sulphate conjugation.

In the first paper on this topic published in 1955, Hilz and Lipmann[20] were able to show that active sulphate probably was an adenylic acid derivative, tentatively identified as adenosine monophosphate sulphate, APS (Fig. 1).

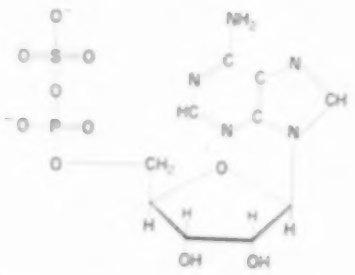

FIG. 1. Structure of adenosine-5′-phosphosulphate (APS).

Continued work on the identification of active sulphate by Robbins and Lipmann[21] in 1956, however, showed that active sulphate obviously contained two phosphate groups and one sulphate group on each adenosine. Mainly on the basis of data obtained by acid and enzymatic hydrolysis, active sulphate was eventually identified as adenosine-3′-phosphate-5′-phosphosulphate, PAPS (Fig. 2).

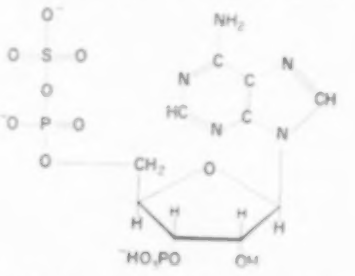

FIG. 2. Structure of 3-′phosphoadenosine-5′-phosphosulphate (PAPS).

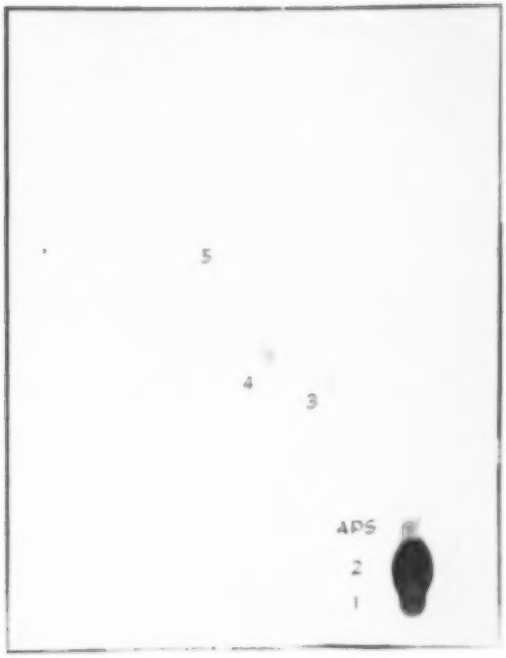

FIG. 5(a). Chromato-autoradiogram showing basic pattern of ^{35}S-labelled spots of incubating medium used in sulphatation experiments *in vitro*. (Rat liver supernatant, ATP. Mg-ions and ^{35}S-labelled sulphate.) (1) and (2) denote inorganic sulphate + 3′-phosphoadenosine-5′-phosphosulphate (PAPS). APS denotes adenosine-5′-phosphosulphate. (4), (3) and (5) correspond to unknown estersulphates formed from acceptors present in the liver preparation.

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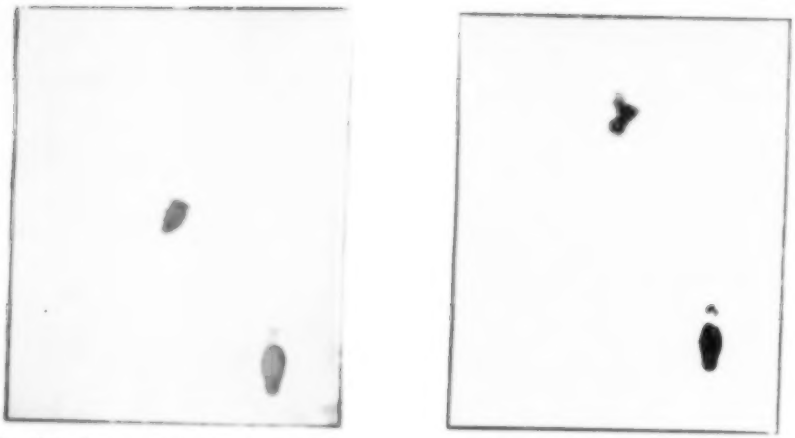

FIG. 5(b). Same as 5(a) after incubation in the presence of phenol (left) and oestron right).

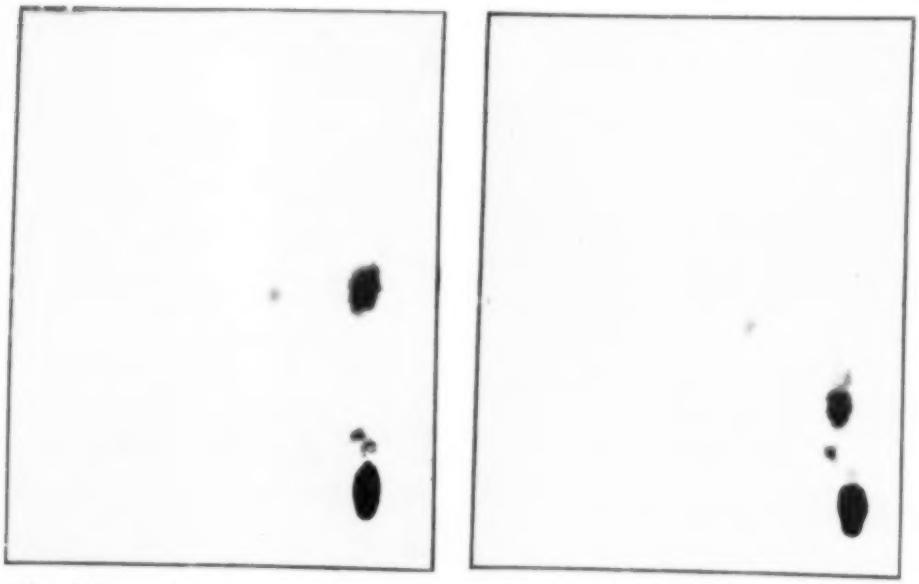

FIG. 6. Same as 5(a) after incubation in the presence of adrenaline (left) and noradrenaline (right).

The demonstration of two differently linked phosphates in the molecule, which seemed to indicate a two-step reaction in the formation of active sulphate was in good agreement with some important results obtained by Wilson and Bandurski[22] in simultaneous and independent work. Their experiments made on yeast showed that the sulphate activation system could be split into two fractions inactive by themselves, but active when combined. These findings were also confirmed by Robbins and Lipmann and some further work on these systems revealed the present conception of the phase of sulphate activation which is summarized in Fig. 3.[23] In a first step catalysed by ATP-sulphurylase, adenosine monophosphatesulphate and pyrophosphate

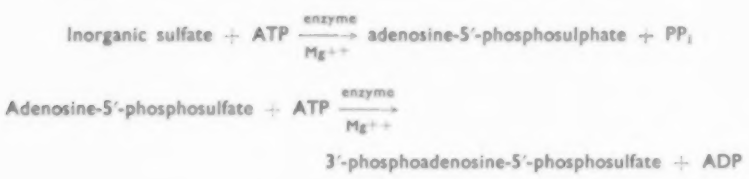

FIG. 3. Sulphate activation.

are formed from sulphate and ATP. In the next step catalysed by APS-kinase, active sulphate and ADP are formed from APS and ATP. The overall reaction thus requires 2 moles of ATP for each mole of active sulphate formed. Both enzymes concerned require as cofactors Mg ions or other divalent cations.

Through this work, the first phase in sulphate conjugation, sulphate activation, may be considered as essentially clarified. The mechanisms described seem to be involved in the formation of all known types of conjugated sulphates.

Our knowledge of the second phase of sulphate conjugation involving transfer of the sulphate group from the activated sulphate to the various compounds concerned is, however, more incomplete.

From the pioneering work quoted earlier, it was obvious that cell-free liver preparations contained enzymes able to effect the sulphate transfer to the ordinary phenols.

In 1955, de Meio and Lewycka[24] were also able to demonstrate the probable formation of dehydro*iso*androsterone sulphate under the same experimental conditions as used in their experiments on phenol sulphate conjugation. In extended work on steroids by Roy[25], Schneider and Lewbart[26] and de Meio *et al.*[27], more direct evidence of the synthesis by liver preparations of sulphates of these and many other steroids such as oestrone, oestradiol, testosterone, etc., were presented.

Steroid sulphate formation by the liver preparation also required Mg ions and ATP in the media and also for other reasons phenol sulphate conjugation and steroid sulphate conjugation seemed to be identical. Thus the recombination of the two activating and transferring enzyme preparations, described earlier by de Meio and coworkers, was necessary for steroid sulphate formation.

It could also be shown that sulphate conjugation of steroids was partially inhibited by the presence in the medium of phenols. The demonstration of this inhibition seemed to indicate a competition between the phenol and the steroid for the same

enzyme system. Another interpretation of the latter observations might, however, be that the two types of substrates are competing for active sulphate but that the transferring enzymes involved in the conjugation of phenols and steroids, respectively, are different.

This conception of the occurrence of a series of sulphate-transferring enzymes, sulphokinases, which are responsible for the sulphate transfer to the various acceptors concerned is now generally accepted. Strongly supporting this view was the fact that Nose and Lipmann[28] were recently able to separate the transferring enzyme of rat liver into three different sulphokinases by means of electrophoresis as shown in this slide. One of these enzymes, dehydroandrosterone sulphokinase, also reacted with progesterone and *iso*androsterone which all have a 3′-β-hydroxyl, and it therefore appears to be a 3′-β-hydroxy steroid sulphokinase.

Another fraction, estrone sulphokinase, was also found. This one was partially separated from a third type, the phenol sulphokinases, responsible for sulphate transfer to a large number of phenols and phenol-derivatives.

In addition to the different transfer enzymes mentioned which thus have been preliminarily separated, other similar sulphokinases involved in the biosynthesis of sulphomucopolysaccharides and cerebroside sulphates may also occur. Studies on the formation of these compounds have been made in Lipmann's laboratory[28, 29] and evidence has been presented which indicates that in both cases sulphurylation follows the general scheme we have been discussing, including sulphate activation (Fig. 3) and sulphate transfer (Fig. 4).[30]

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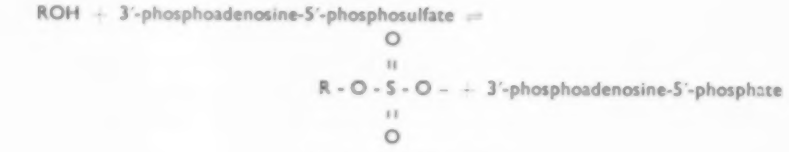

FIG. 4. Sulphate transfer.

After this review on the enzymatic mechanisms involved in sulphate conjugation, we can now consider for a moment another point of interest in this connexion, i.e. the nature of compounds which are conjugated with sulphate in mammalian tissues.

It seems to have been generally believed, until some 20 years ago, that the only hydroxy compounds which form sulphate conjugates on administration to mammals are phenols. The discovery of various steroid sulphates, among them also several sulphates of nonphenolic steroids, seemed to necessitate a revision of this view.

In a short communication in 1948, Anderton *et al.*[31] claimed that two things were necessary for sulphurylation of an OH group *in vivo* (1) $10 > pK_a > 7$. (2) The OH in an enolizing system.

More recently, however, Roy[25], basing his view on the successful sulphurylation of several steroid alcohols by liver enzymes *in vitro* and the known occurrence of some of these compounds in urine, suggested that "the claim of Anderton and coworkers was an oversimplification of the situation and that the ability of any particular hydroxyl group to conjugate with sulphate is not governed only by p*K* value, but also by the structure of the molecule as a whole".

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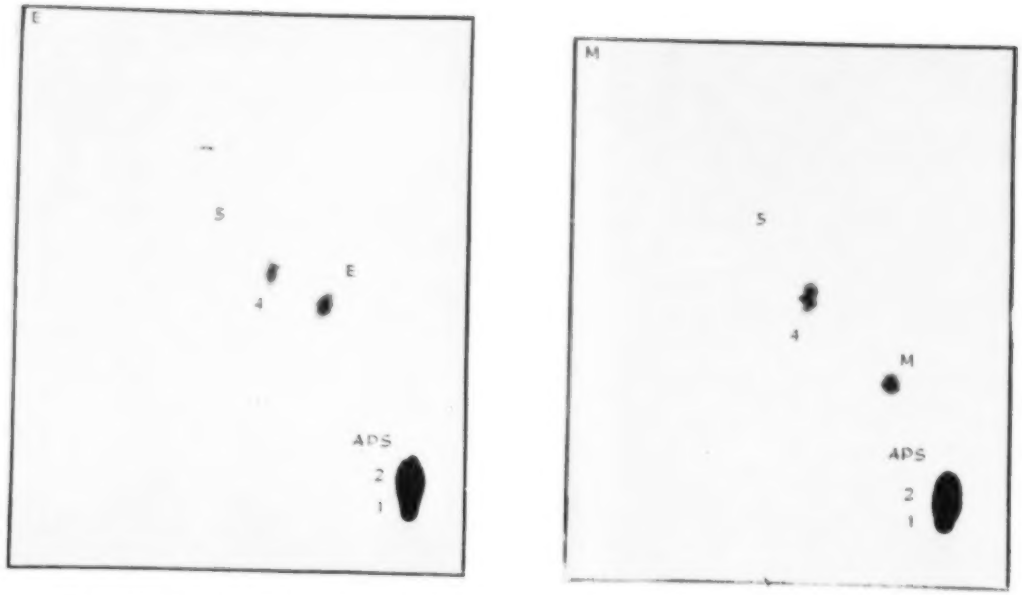

FIG. 7. Same as 5(a) after incubation in the presence of ethanol (left) and methanol (right). Spots *E* and *M* correspond to ethanol sulphate and methanol sulphate, respectively.

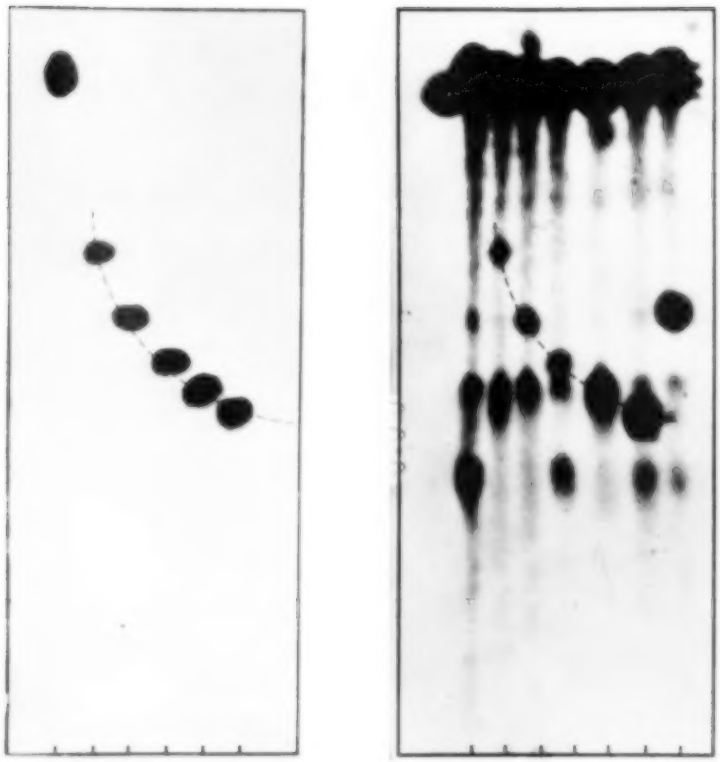

FIG. 8. Electrophero-autoradiograms of ^{35}S-labelled methanol, ethanol, propanol, butanol- and amylol sulphates (indicated by the curved lines) prepared by organic synthesis (left) and biosynthesis (right).

At present the family of possible sulphate acceptors seems to be rapidly increasing due to the availability of new and sensitive methods for their detection. Until a more detailed picture of the nature of possible sulphate acceptors *in vitro* has been obtained, it seems to be rather difficult to tell why certain OH compounds conjugate with sulphate while others do not. The fact that the border line between possible sulphate acceptors and non-acceptors obviously is not a very sharp one seems to make this task more difficult at present.

There is, however, a more important problem than the question about the type of chemicals which can be conjugated with sulphate in various tissue preparations *in vitro*, from both the biochemical and physiological points of view. I refer to problems concerning the nature of the ester sulphates which under normal or pathological conditions occur in tissues and body fluids. In this respect the present knowledge is very limited. Although, a few normally occurring sulphates of phenol- and indole-derivatives, such as phenol, *p*-cresol, tyrosin, indoxyl and several of the steroid sulphates have actually been isolated from various mammalian urines, much more information on the details of the normal ester sulphate patterns of body tissues seems desirable.

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Since so many important metabolic processes in the mammalian tissues are in one way or another related to sulphate conjugation, it seems reasonable that variations of these patterns not only reflect the putrefaction processes of the gut, which were the main interest in the early studies of this topic, but also many other normal or abnormal functions.

In order to obtain some new data on possible sulphate acceptors and on the ester sulphate patterns of various body fluids we started some time ago a study on these topics. In the following we would like to exemplify some of our results by showing a series of autoradiograms.

In all our experiments which were made either *in vitro* or *in vivo* ^{35}S-sulphate was used as tracer substance. The *in vitro* studies[32] were performed by incubation of the various compounds of interest in a sulphurylating system containing particle-free extracts from liver or other tissues in addition to ATP, Mg ions and radioactive sulphate. Similar experiments with tissue slices have also been made. High-voltage electropherograms and two-dimensional paper chromatograms were run on 5 μl. samples of the incubating media and then applied to autoradiography.

Fig. 5(a) shows the basic pattern of the particle-free sulphurylating liver system incubated for 2 hr on its own, without any added sulphate acceptor.

A few very characteristic spots appear. The pattern in the bottom right corner close to the starting point consists of inorganic sulphate and active sulphate which are not separated in this system, and above this spot adenosine monophosphosulphate can usually be seen. In addition, two or three other spots appear, obviously due to sulphurylation of compounds present in the liver extract. The nature of these compounds has not been established so far but we have some reason to believe that one is a phenol sulphate and another one is a steroid sulphate.

If a suitable sulphate acceptor is incubated in this *in vitro* system, sulphurylation would occur according to the earlier work just reviewed. This would give one or several new conjugated sulphate spots on our autoradiograms which most probably correspond to conjugated sulphates of the compounds added.

Fig. 5(b) shows the pattern of the incubating medium if phenol (left) is added to the sulphurylating system.[33] In addition to the basic pattern shown in the former figure, a new distinct spot of phenol sulphate appears in a typical position. Fig. 5(b) (right) shows the same type of experiment run with oestron. Even in this case, a new spot of oestron sulphate appears on our autoradiograms.

The same type of results as shown in these two slides were of course obtained with all the other well known sulphate acceptors among phenols and steroids we have tested. It was more interesting, however, to apply these techniques to other compounds where data on sulphate conjugation are incomplete or lacking.

Physiologically occurring catechol-type pressor amines belong to this group of substances. It now seems to be generally accepted that adrenalin and noradrenalin are detoxicated, partially by sulphate conjugation. So far, however, demonstration of sulphate conjugation of these two ortophenolic compounds has not been made *in vivo*. This view is mainly supported by the fact that sulphate conjugates of some related compounds, e.g. epinin after administration to mammals, have been isolated from the urine, as reported by Richter[34]. *In vitro* experiments on liver slices reported by Sato *et al.*[35] also favour this opinion.

If adrenalin is incubated in a sulphurylating medium two new spots appear on our autoradiograms (Fig. 6, left).[32] One of them, close to the inorganic sulphate spot, is fast moving in electrophoresis. The other one moves more slowly on electrophoresis and it seems reasonable to suggest that the former corresponds to a disulphate of adrenalin and the second to the monosulphate. Somewhat similar pictures were obtained with noradrenalin. Even in this case, two new spots appeared (Fig. 6, right).

Another observation of some interest made in another series of *in vitro* experiments with the particle-free sulphurylating was the following.

In some of our *in vitro* experiments on steroids and other compounds insoluble in water, the substance to be tested was dissolved in ethanol before the addition to the particle-free sulphurylating system, a method earlier used by other workers in this field. We found, however, that in all those experiments where ethanol was added, a dramatic change in the basic pattern of the autoradiographs took place.

As seen in Fig. 7 (left), a marked increase of a spot designed as no. (3) occurred in addition to a weakening of the ordinary main spot designed as no. (4). This phenomenon occurred independent of the nature of the substance dissolved in ethanol.

Since aliphatic alcohols through the work of Neubauer[36] in 1901 and by Kamil *et al.*[37–39] in 1952 and 1953 are known to conjugate with glucuronic acid, we hazarded the opinion that this new spot was, in fact, identical with a sulphate conjugate of ethanol. This possiblity has been discussed in earlier literature, but no evidence in support for this view has been presented so far. We therefore extended our experiments to other alcohols and each alcohol tested induced the formation of a new spot characteristic to the alcohol.[40] The methanol-induced spot migrated somewhat more slowly in both solvents than the spot obtained from ethanol, as shown in Fig. 7 (right). Control experiments showed that these compounds were not formed in the absence of ATP or with boiled liver extract.

In order to get further support for our suggestion that these compounds were in fact ester sulphates of aliphatic alcohols, ^{35}S-labelled specimens of these five alcohol sulphates were prepared by organic synthesis in the usual way starting from concentrated sulphuric acid and the alcohols concerned.

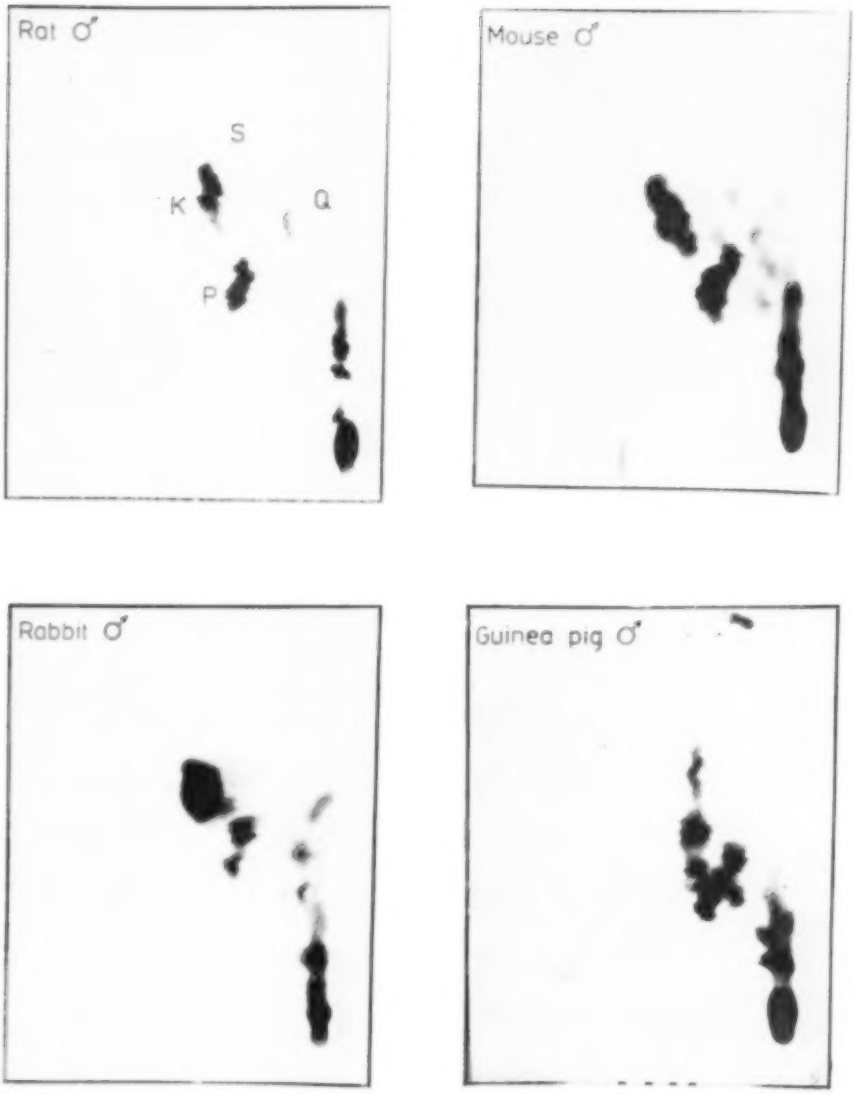

FIG. 9. Chromato-autoradiogram showing the urinary "ester sulphate pattern" of different species (males) after injection of ^{35}S-labelled sulphate.

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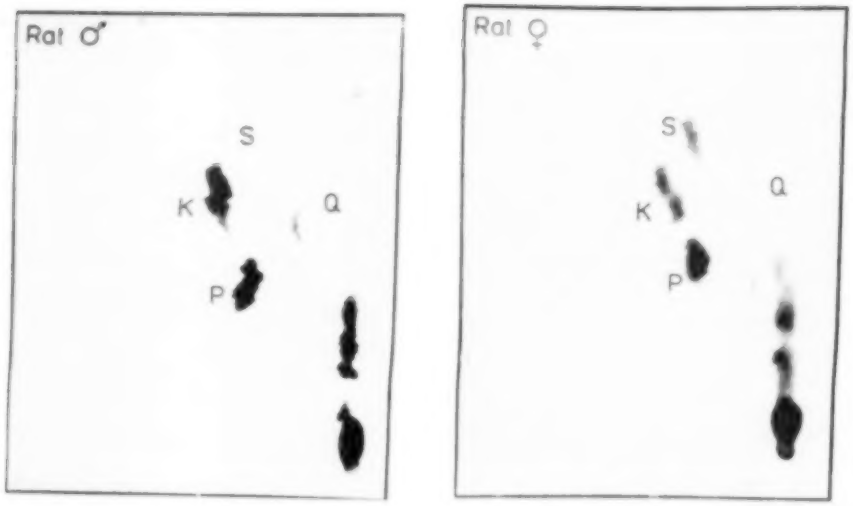

FIG. 10. Urinary ester sulphate patterns of male (left) and female (right) rats. The female rat spot (*S*) is absent in male rats.

The synthetic aliphatic alcohol sulphates were found to migrate to the same positions as those compounds formed by enzymatic synthesis on our two-dimensional paper chromatograms and also as shown in Fig. 8. The left part of this figure shows the localization of the synthetic methanol sulphate, ethanol sulphate, propanol sulphate, etc., as indicated by the curved line. The right part of the figure summarizes a series of *in vitro* experiments made with corresponding series of alcohols present in the sulphurylating medium.

From these experiments we conclude that liver extract contains enzymes catalysing alcohol sulphate conjugation. Extended work on this line has shown that sulphate conjugation *in vitro* is not limited to monohydric aliphatic alcohols. As shown in another report from our laboratory, certain polyols are also possible sulphate acceptors.[41]

The last point we would like to make deals with the ester sulphate patterns of body fluids of mammals as studied by means of two-dimensional paper chromatography combined with autoradiography. In all the *in vivo* experiments which will be mentioned, the experimental animals were given intravenous or intraperitoneal injections of radioactive sulphate in the order of 2–3 mc/kg of body weight. The animals were then kept for 24 hr under fasting conditions with water *ad libidum* in metabolic cages for urine collections. Other body fluids studied were obtained in connexion with the slaughter of the animals. Samples of 5 μl of the body fluids concerned were applied without any pretreatment to paper chromatography.

The ester sulphate patterns of sex mates of four different species were investigated with this technique.[42] In general, the differences in the patterns of individuals belonging to the same sex and species were small. Also the different species showed great similarities as seen in Fig. 9. Most originality was shown by the guinea pig which has a typical spot above the region designed as *K* in the rat pattern and another one close to the *P*-region. Unfortunately, we don't know very much about the nature of all these spots except that they are easily hydrolysed by acids and fairly resistant to alkali. From this fact in addition to the well known circumstance that radio-sulphur given as sulphate is mainly incorporated in ester sulphate compounds, we may conclude that all these compounds are probably ester sulphates. On the basis of our *in vitro* studies we can also mention that cresol sulphates are expected to appear in the *K*-region, phenol sulphate and indoxyl sulphuric acid in the *P*-region and most of the steroid sulphates in the *S*-region which appears fairly empty in the urines of these creatures we tested. In female rats, as shown in Fig. 10, we have a typical spot in this region which is absent in males.

If any further conclusions are permitted on the basis of the somewhat immature studies exemplified in the last few figures, we would like to say that the ester sulphate patterns of the urines under standard conditions, judging from the small individual variations found, is a rather typical biochemical feature in mammals.

The series of autoradiograms shown in Fig. 11 bring us back to the sulphate conjugation of aliphatic alcohols. In order to see whether or not sulphate conjugation of alcohols takes place even *in vivo*, a series of rats was given 2 ml of 40% solutions of the earlier mentioned alcohols by gastric tube simultaneously with the injection of radioactive sulphate.[43]

In the urine pattern of the methanol rats, a new spot, chromatographically and electrophoretically identical with methyl sulphate appeared (Fig. 11, *M*), which was

absent in the patterns of normal rats (Fig. 11, *N*). In the urine of the ethanol treated rats, the characteristic ethanol sulphate spot appeared, etc. (Fig. 11, *E*).

From these experiments we conclude that aliphatic alcohols are partially excreted in rats as alcohol sulphates.

The evaluation of the physiological role of this route of detoxication of alcohols seems rather difficult until more quantitative data on alcohol sulphate conjugation are available. On the basis of the large number of autoradiograms evaluated it seems, however, likely that the alcohol sulphate in the urine of alcohol-treated animals represents a considerable portion of the total ester sulphate fraction.

It is fairly well known, e.g. through the extensive studies of Bray *et al.*[44] in 1952 that the amounts of sulphate available for sulphate conjugation are limited and are known to decrease markedly under fasting conditions or periods of low protein intake. Consequently, the possibility seems to exist that an excessive alcohol intake during a period of protein depletion might, due to competition, disturb the detoxification of other toxic compounds by sulphate conjugation.

Fig. 12 illustrates the fate in a rat after intraperitoneal injection of ^{35}S-labelled ethyl sulphate, prepared by chemical synthesis.[45] Collections were made at 2, 4, 7, and 24 hr.

It is seen that the bulk of the radioactivity in the urine corresponds to ethyl sulphate. A slow liberation of sulphate occurs, however, somewhere within the body and the liberated sulphate is again available for conjugation at least with some of its normal acceptors, probably paracresol, phenol and indoxyl. Obviously, a considerable part of the ethyl sulphate given is excreted unchanged, a finding that at least does not contradict the opinion that sulphate conjugation may play a role in detoxification of alcohols.

The full elucidation of the physiological significance of this process as many others in the field of sulphate conjugation, which was opened to us by Baumann and his colleagues some 80 years ago, still lies ahead in the future.

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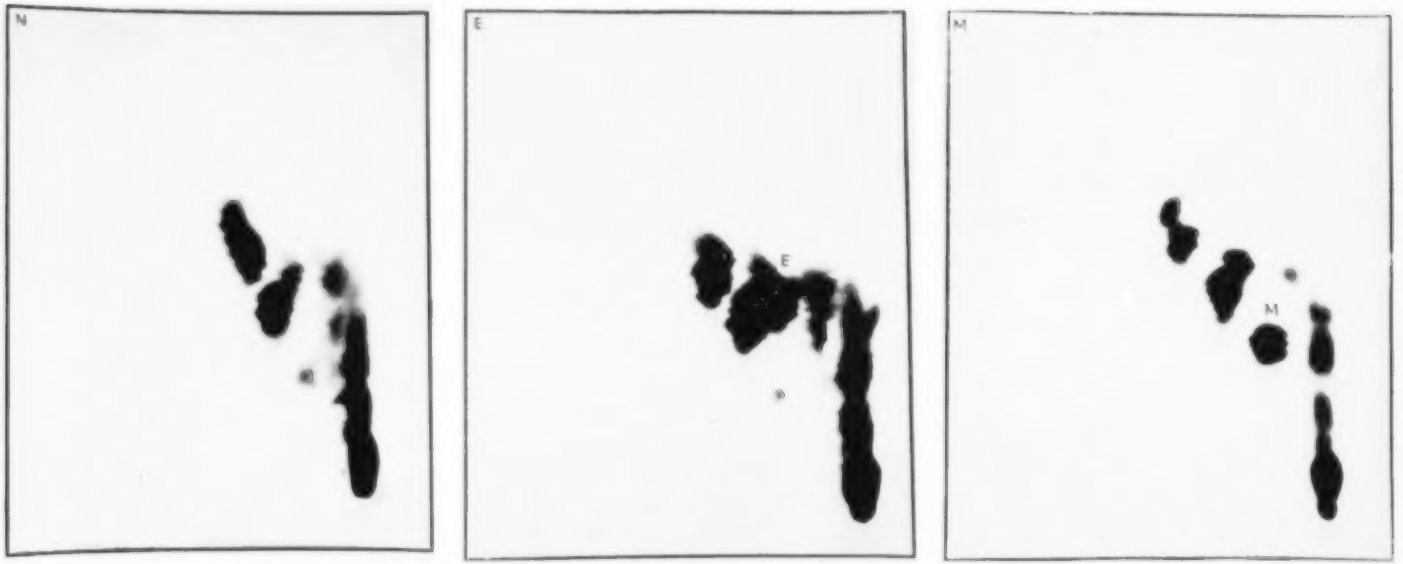

FIG. 11 Urinary ester sulphate pattern of normal rat (*N*), ethanol treated (*E*) and methanol treated (*M*) rat. Spots denoted as *E* and *M* correspond to ethanol- and methanol sulphate, respectively.

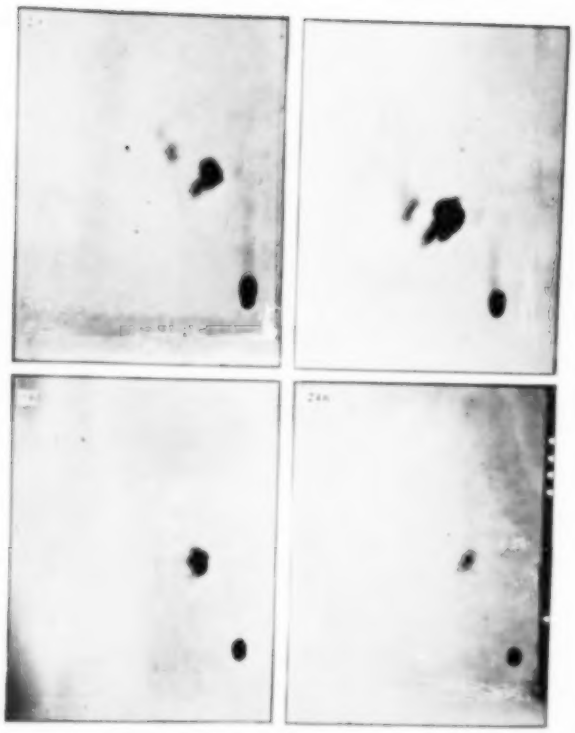

FIG. 12. Urinary ester sulphate pattern of a rat injected with ^{35}S-labelled ethanol sulphate 2, 4, 7 and 24 hr after injection.

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EXPERIMENTAL STUDIES OF GASTROINTESTINAL CONJUGATION FUNCTIONS

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Abstract—Experimental (duodenal) ulcer can be caused by feeding excessive amounts of cinchophen to dogs. Studies on the mechanism of ulcer formation revealed the importance of conjugation systems since it was possible that cinchophen depleted the liver of glucuronic acid. The ability of liver slices taken from cinchophen intoxicated animals to carry out glucuronide synthesis necessitated a search for other organs capable of carrying out glucuronide conjugation reactions. Negative results were obtained with a wide number of tissues and apart from liver and kidney only slices taken from the mucous membrane of stomach, duodenum, ileum and colon gave high values for glucuronides synthesis. Other results showed that a number of substances were conjugated in the gastrointestinal tract; the significance of these results in relation to ulcer formation is discussed.

THE writer became interested in the detoxication mechanisms and specifically on the glucuronide formation in connexion with our previous studies of the mechanism of the experimental cinchophen (atophan) ulcer. It has been shown by van Wagoner and Churchill[1] that excessive feeding of cinchophen to dogs results in ulcer formation in the pyloric and duodenal regions. This method is one of the most reliable ways of producing an experimental ulcer.

In our own studies we were able to show that cinchophen causes a rather specific reduction and even cessation in the mucus secretion by the pyloric and duodenal (Brunners) glands[2, 3] in dog. The same was shown to be true also for the secretion by the pyloric glands.[4] Mucoproteins, which are secreted in the mucus, contain glucuronic acid and our interest was aroused by the fact that cinchophen is excreted and detoxicated as a glucuronide conjugate. According to the ideas of most authorities at that time—I would like to refer to the monograph of Williams[5] glucuronide conjugation is carried out by the liver and to a lesser extent by the kidney. The possible depletion resources of glucuronic acid of the liver by cinchophen feeding was the first working hypothesis. It was, however, found, that the whole organism[6] as well as liver slices taken from cinchophen toxicated animals[7, 8] were capable of carrying out glucuronide synthesis even after ulcers had developed in the same animal.

The next step in our work[9, 10] was to find out whether the liver and kidney really were the only important sites for the glucuronide conjugation reactions, or was it possible that other organs could also participate in this activity.

The studies were conducted in *in vitro* conditions. Tissue slices were incubated in the Warburg apparatus in the presence of *o*-aminophenol. The glucuronide conjugate of

this compound is easy to detect by colour reactions and can be quantitatively assessed according to the method described by Levvy and Storey.

Table 1 gives the results produced by liver and kidney slices taken from various animal species.

TABLE 1. GLUCURONIDE SYNTHESIS OF THE LIVER AND KIDNEY IN VARIOUS ANIMAL SPECIES. γ *o*-AMINOPHENOL CONJUGATED (100 MG DRY WEIGHT OF TISSUE) 90 MIN

Organ	Animal	Range	Mean
Liver	Rabbit	38–236	123
	Rat	24–195	65
	Guinea pig	69–269	124
	Dog	40–110	66
	Cat	trace?	—
Kidney	Rabbit	38–138	88
	Rat	24–156	79
	Guinea pig	43–96	80
	Dog		
	Cat	trace?	—

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TABLE 2. LIST OF ORGANS TESTED FOR GLUCURONIDE SYNTHESIS

Organ	Animal	Results
Pancreas	Rabbit, rat, cat, guinea pig	negative
Spleen	Rabbit, rat, dog, guinea pig	negative
Adrenals	Rabbit, rat, dog, guinea pig	negative
Ovary	Rabbit, rat, dog, guinea pig	negative
Testicle	Rabbit, rat	negative
Placenta	Rabbit, rat, human	negative
Peritoneum	Rabbit, rat	negative
Muscle		
diaphragm	Rabbit, rat	negative
skeletal	Rabbit, rat	negative

TABLE 3. GLUCURONIDE SYNTHESIS. LIST OF ORGANS KNOWN TO CONTAIN OR PRODUCE GLUCURONIC ACID AND TESTED FOR THEIR ABILITY TO CONJUGATE *o*-AMINOPHENOLGLUCURONIDE *in vitro*

Organ or tissue	Animal	Result
Synovial membrane	Rabbit, rat	negative
Umbilical cord	Rabbit	negative
Excised eye, various parts	Rabbit, rat	negative
Trachea, mucous membrane	Rabbit, rat	negative
Ureter, mucous membrane	Rabbit, rat, guinea pig	negative
Urethra, mucous membrane	Rabbit	negative
Urinary bladder, mucous membr.	Rabbit, rat, dog, cat	trace?

Table 2 shows the negative results obtained with a variety of other organs. In the next series (Table 3) those organs were tested which either produce glucuronic acid containing compounds (e.g. hyaluronic acid) or which otherwise contain it. As can be seen, the results were negative.

The studies were then continued by taking slices from various parts of the alimentary canal. The results are listed in Table 4. It can be seen that slices taken from the mucous membrane of the stomach, duodenum, ileum and colon all gave markedly high values for glucuronide synthesis. These findings are thus in agreement with some earlier work which indicate that the sulphate conjugation takes place also in the gastrointestinal tract.[11, 12]

TABLE 4. GLUCURONIDE SYNTHESIS BY THE MUCOUS MEMBRANE OF THE GASTROINTESTINAL TRACT. *o*-AMINOPHENOL CONJUGATED (100 MG DRY WEIGHT TISSUE) 90 MIN

Organ	Cat	Dog	Rabbit	Rat
Stomach				
greater curvature	—		10–100	62– 71
lesser curvature	—		25–217	–150
pyloric	—	32–145	69–240	30–511
Duodenum	—	55–160	28–568	48–567
Ileum	—	–175	82–328	40–208
Colon	—		29–300	27–657

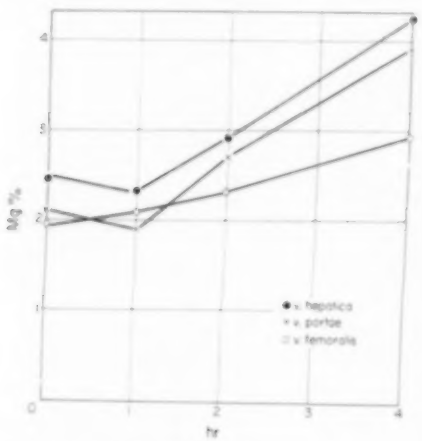

FIG. 1. Serum glucuronides after intragastric feeding of 500 mg cinchophen in dog. D. choledochus was ligated.[17]

Independently of these studies, and at the same time, two other groups had arrived at the same conclusion. Zini[13] and Shiray and Ohkubo[14] reported that significant glucuronide synthesis occurs in adult stomach and intestine. All these studies were later confirmed by Dutton[15, 16] who was also able to demonstrate the presence of the same enzyme machinery in the mucosal elements.

All these studies have been performed with isolated tissue slice techniques, We have also performed studies in whole animals. The portal blood, venous hepatic blood and systematic blood were analysed after feeding of a glucurogenic substance, phenolphtalein or cinchophen, to dogs. It was found (Fig. 1) that the glucuronide content in

the portal blood rose by approximately 100 per cent, even when the escape of bile into the intestine was prevented by ligation of the common bile duct.[17]

Similar studies have been performed for foetuses.[18] Intragastric feeding of phenolphtalein phosphate to nearly full term guinea pig foetus *in utero* resulted in the appearance of easily detectable amounts of phenolphtalein glucuronide both in the foetal and maternal blood (Fig. 2).

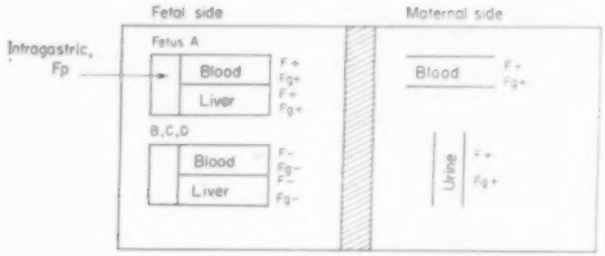

FIG. 2. Studies of the intrauterine gastric feeding of 250 mg of phenolphtalein phosphate (Fp) in the guinea pig. F, free phenolphtalein, Fg, phenolphtalein glucuronide.[18]

We have started a series of experiments in order to find out which kind of substances undergo glucuronide conjugation in the gastrointestinal tract. Some of the results are listed in Table 5.[19–21] In these studies the substances were incubated with the tissue slices, after which the substrate and metabolites were extracted with specific solvents. The final identification was then performed by chromatographic analyses.

TABLE 5. DUODENAL GLUCURONIDE SYNTHESIS

Compound	Glucuronide formation
Estrone	+
Estriol	+
Estradiol	+++
Equilin	++
Stilboestrol	++
Progesterone	–
Pregnandiol	–
Androsterone	–
Testosterone	–

As can be seen (Table 5) only those steroids which possess oestrogenic activity are conjugated, whereas the androgens are not. All the steroid hormones tested so far and which have been found to be conjugated to glucuronides contain a three carbon phenol hydroxyl in their molecule. None of the unconjugated steroids have this characteristic. This appears to hold for the other steroids tested so far. Thus neither cortisone or hydrocortisone are conjugated by the mucous membrane. Our present work is also partly concentrated on the probable molecular specifity of the glucuronide conjugations.

Since it has been well established that the glucuronide conjugation is not solely confined to the liver and kidney but that it is also carried out by the gastrointestinal

tract, we are left to discover the relationship between the hepatic and intestinal synthesis, and the physiological meaning of these processes in the gastrointestinal tract.

As to the behaviour of the hepatic and intestinal processes under varying experimental conditions Table 6 illustrates few of the results obtained so far.[22–24] It can be seen that the hepatic and duodenal conjugation are not affected in the same way by the same factors. This can also be seen in Figs. 3 and 4. In these studies a 400 r or 1200 r

TABLE 6. EFFECT OF VARIOUS AGENTS ON GLUCURONIDE SYNTHESIS

Agent added *in vivo*	Intestinal synthesis	Hepatic synthesis
Thyroxin	*	+
I^{131}	*	—
Cortisone	—	*
Cinchophen	*	*
Carbontetrachl added *in vivo*	*	—
Heparin	*	*
Histamin	*	*
Serotonin	*	*
"Intrinsic factor"	*	*
B_{12}	*	*

* = no effect.
\+ = stimulation.
— = depression.

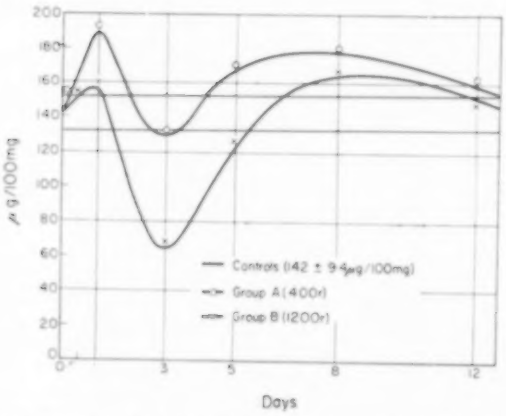

FIG. 3. Effect of x-ray irradiation on the *o*-aminophenolglucuronide synthesis by the liver parenchyma. Results expressed as ug *o*-aminophenolglucuronide per 100 mg dry weight liver tissue.[26].

x-ray dose has been applied locally to the exposed liver or stomach, after which the synthetic capacity was measured at certain intervals.[25, 26] It appears that the liver conjugation mechanism is rather resistant towards irradiation compared with the gastric mucous membrane. In both cases the effect is, however, reversible.

What is the meaning of the gastrointestinal conjugations? The answer cannot be given yet, though several possibilities are at hand. First, that it is a true detoxication

reaction by which organic compounds are made less toxic or active. Another alternative is that these reactions serve the purpose of active transport across cellular membrane. It is known that for many substances the conjugation leads to a better solubility in water and biologic fluids. If so, then these reactions would actively help the selective adsorption from the gastrointestinal tract. For many of these substances diffusion in the free state, rather than selective absorption in the conjugated form, seems to be

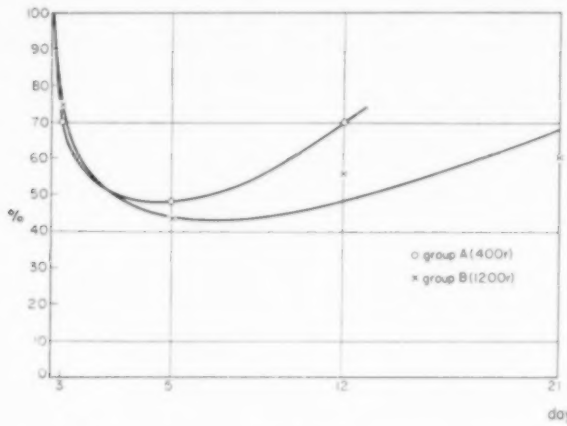

FIG. 4. Effect of x-ray irradiation on the *o*-aminophenolglucuronide synthesis by the gastric mucous membrane. Results expressed as percentage changes from the simultaneous control values.[25]

more likely. I would like to refer to the work of Schachter who has found that after feeding of salicylates the resulting concentration of unconjugated salicylate in the plasma was 100–200 times the concentrations of the salicyl glucuronides. More experimental work is needed to answer these questions.

Even so, there are some aspects which already might be of interest for us.

When performing synthetic reactions in general one part of the molecule synthetized is provided by the organism—in this case glucuronic acid. When the dose of the foreign compound is not excessive the conjugating agent may be provided from waste material or from the tissues, without strain upon the resources of the animal. With excessive doses, however, the conjugating agent may be utilized for detoxication at the expense of material required for the well-being of the organism. This has been illustrated by the case of bromobenzene,[5] which when fed to growing animals in excess of a certain dose, causes cessation of growth, apparently because the animal is unable to provide sufficient cysteine for both growth and the detoxication of the excessive amounts of bromobenzene. A similar condition seems to exist during glucuronide conjugation. It was actually the side-effect, i.e. the depletion of the mucoprotein production by the mucous membrane, which led us to the discovery of the local gastrointestinal glucuronide synthesis. Two competitive functions, both carried by the cellular components of the mucous membrane, may be taking place simultaneously. With excessive doses of cinchophen, for example, mucous secretion is inhibited. One function of the mucus is to protect the epithelial surface against chemical and mechanical

injuries. When this protection is lacking other consequences may follow. In the case of excessive doses of cinchophen the result may be erosions or even actual ulcer formation. If this hypothetical line of events actually takes place, we would then be able to explain and understand some of the frequent gastrointestinal manifestations provoked by many of our drugs. I would particularly like to draw attention to the gastrointestinal side-effects, including ulcers, caused by the widely used salicylates. It has been claimed[27] that the carboxyl and phenol groups of salicylate are conjugated with glucuronic acid by intestine slices.

As to the probable competition with some other functions in the gastrointestinal tract, it appears that simultaneous administration of a glucurogenic substance, e.g. cinchophen, does not interfere with the absorption rate of glucose. On the other hand glucose seems to enhance the absorption of a glucurogenic substance.

We have also been quite concerned about the role of β-glucuronidase in the conjugation processes. An experimental condition was sought which would allow parallel analyses of the glucuronide conjugation and β-glucuronidase activity of the same

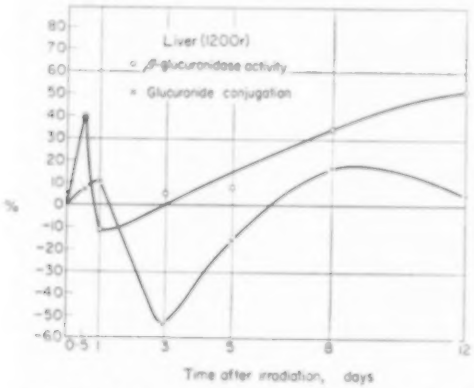

FIG. 5. Comparison of the hepatic β-glucuronidase activity (○–○) and the glucuronide conjugation capacity (×–×) after a local 1·200 r x-ray dose.[28]

tissue when changes are caused in the former. In our previous x-ray irradiation studies we had an opportunity of causing constant changes in the synthetic activity. These experiments were continued, and from the results (Figs. 5 and 6) it is evident that the β-glucuronidase activity curves are different in the two organs after the irradiation. In the stomach the activity parallels the simultaneous glucuronide synthetic capacity, whereas in the liver, there is a rise and depression of the enzyme activity similar to the glucuronide conjugation capacity. Nevertheless there is no direct relationship between these two functions. There is a certain time lag in the β-glucuronidase curve that the maximum and minimum points of the two curves do not coincide with each other.

As to the question of the possible role of β-glucuronidase in the synthetic reactions, these results may be taken as an indication there is no such relationship.

We are still not satisfied with our present knowledge of the physiological role that β-glucuronidase plays in the conjugation processes. We have tried to attack this

problem from another angle. We turn back to our original gastric lesion problems. Fig. 7 shows the preliminary results which have been obtained after attempts to inhibit the cinchophen effect in animal.[29] Chicks have been chosen for trials first because cinchophen also causes easily detectable gastric ulcers in this species, and secondly because it is a cheap animal which permits the use of large-groups.

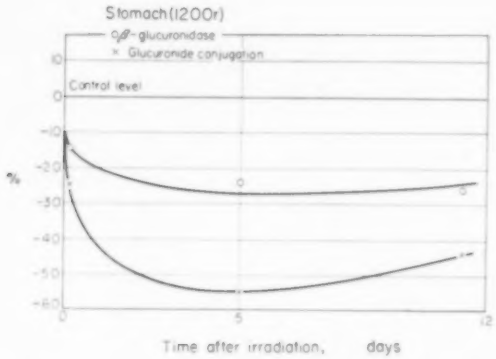

FIG. 6. Comparison of the β-glucuronidase activity (○–○) and the glucuronide conjugation capacity (×–×) of the gastric mucosa after a local 1·200 r x-ray dose.[28]

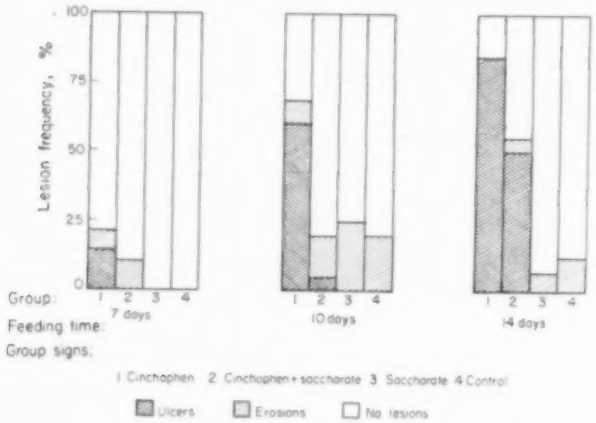

FIG. 7. Effect of saccharate on the frequency of the cinchophen provoked gastric lesions in chicks.[28]

We have administered a β-glucuronidase inhibitor, saccharic acid lactone in connexion of the cinchophen feeding. It can be seen that simultaneous administration of this inhibitor with the glucurogenic substance, cinchophen is able markedly to reduce the ulcer frequency. At the 10 day point there are only 8 per cent with ulcers in this group as compared to 65 per cent in the group without the inhibitor. On the fourteenth day the same figures are 50 per cent against 85 per cent. I want to stress that these are only preliminary studies. More work is needed for control and understanding of the immediate complex mechanism underlying these effects. We know that the stomach of the chick is able to synthetize *o*-aminophenol and there is also large

amounts of β-glucuronidase in it. These experiments may be taken as a further indication that the cinchophen ulcer might be related to the glucuronide formation machinery and that the competitive functions between the glucuronide synthesis and the mucopolysaccharide involving protective mucous production could be involved in these processes.

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PREPARATION AND FRACTIONATION OF MUCOPOLYSACCHARIDES

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Abstract—Techniques are described for the preparation and fractionation of polysaccharides. The breakdown of protein in connective tissues for the release of mucopolysaccharides is best accomplished by the use of proteolytic enzymes. Good recovery of polysaccharides is obtained with this method. Subsequent to proteolytic digestion of tissue, polysaccharides can be recovered from a mixture of amino acids, salts, fats and nucleic acids by subjecting the mixture to successive treatments with alkaline and acid alcohol followed by phenol extraction. Quaternary ammonium compounds may be used prior to alcohol precipitation. Polysaccharide mixtures can be fractionated by zone electrophoresis. The use of chromatographic procedures and of precipitation with different salt concentrations in the presence of quaternary ammonium compounds is also described.

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THE connective tissue is made up of fibrous elements, cells and an amorphous intercellular substance generally called the ground substance. The ground substance contains polysaccharides.

In Table 1 the polysaccharides isolated from connective tissue are listed.

TABLE 1.

Polysaccharide	Monosaccharide composition
Non-sulphated	
Hyaluronic acid	N-acetylglucosamine glucuronic acid
Chondroitin	N-acetyl-galactosamine, glucuronic acid
Sulphated	
Chondroitinsulphate A	N-acetylgalactosamine, glucuronic acid
Chondroitinsulphate B	N-acetyl-galactosamine, iduronic acid
Chondroitinsulphate C	N-acetyl galactosamine, glucuronic acid
Heparitinsulphate	Glucosamine, glucuronic acid
Keratosulphate	N-acetylglucosamine, galactose

In order to determine the nature and the amount of mucopolysaccharides in a tissue specimen two main principles can be used:

(1) Direct acid hydrolysis of the tissue and analysis of the resulting mixture of monosaccharides.

(2) Extraction and fractionation of the polysaccharides and determination of their chemical and physical properties.

The first method does not give any detailed information regarding the polysaccharide pattern in the tissue. Several polysaccharides may exist with all or some of

their building stones in common. The second method will give a more detailed picture of the polysaccharide pattern of the tissue, but being more complicated it is liable to larger errors.

The ideal method for preparation and fractionation of the polysaccharides should allow all polysaccharides to be extracted quantitatively from any tissue and to be quantitatively separated into clear-cut fractions, each consisting of a single chemically and physically individual polysaccharide. Such a method has not to my knowledge been worked out yet.

Most of our knowledge of the polysaccharide pattern derives from the combination of the results obtained by different authors using different methods.

EXTRACTION OF POLYSACCHARIDES FROM TISSUES

At the beginning of this century extraction with alkali was the general method used for the isolation of the polysaccharides from tissues. This method was later on used in most of the early work done by the leading schools in this field like those of P. A. Levene, Karl Meyer, Erik Jorpes and H. Masamune.

The greatest disadvantage in the alkaline extraction is the possibility of degradation of the polysaccharide molecules. Moreover the possibility exists that not all polysaccharides are extracted.

Blix and Snellman[1] showed that the physical properties of CSA* prepared by the alkaline extraction method are different from those given by the polysaccharide extracted with milder methods. On the other hand the possibility exists that the larger molecules obtained by milder methods are complexes between proteins or peptides which cannot be regarded as part of the polysaccharide molecule itself.

Today most investigators agree that the use of proteolytic enzymes for the breakdown of tissue proteins in order to release the mucopolysaccharides gives the best recovery of polysaccharides. Moreover these methods do not seem to degrade the polysaccharides, provided they are not attacked by the enzymes used in the procedure.

As early as 1891 Schmiedeberg[2] used proteolytic enzymes for the preparation of polysaccharides. He used gastric juice. The amount of carbohydrate in the gastric juice was, however, fairly high, and thus contaminated the final product. Later on pepsin, both crystalline and purified, have been used. In order to obtain a satisfactory digestion with pepsin the procedure has to be carried out at an acid pH which may cause degradation of the polysaccharide. It cannot, for instance, be excluded that acid-labile bonds like the sulphaminic group in heparin[3] can be broken during this treatment. When trypsin, chymotrypsin or intestinal enzymes are used, the degradation due to the acid reaction is excluded.

The digestion has been carried out in different ways. Some authors only digest for a short time and thus have the tissue liquefied. This does not necessarily mean that all proteins are broken down in fragments small enough not to interfere with the subsequent purification procedures. In such cases some additional treatment like alkali extraction[4] or a preliminary treatment with pepsin is necessary.[5]

A better method of digestion with trypsin and chymotrypsin is to add small amounts of the enzymes frequently. The course of the digestion can be followed by

*The following symbols are used:—CSA, Chondroitinsulphuric acid; CPC, Alkylpyridinum chloride; CTAB, Cetyltrimethylammonium bromide.

determination of the ratio of amino-nitrogen to total nitrogen. Crude glycerol extract of acetone dried and powdered pig pancreas seems to be much more effective in digesting several tissues than crystalline trypsin and chymotrypsin. The digestion is still better if a glycerol extract of intestinal mucosa is added.[6] This is obviously due to the presence of several peptidases which depolymerize the proteins far better than the pure pancreatic enzymes. This point is very important when the polysaccharides have to be recovered from the solution by means of alcohol precipitation.

It can be argued that the addition of the crude extract may contaminate the final product. However, the amount of extract needed is small and its carbohydrate content rather low. In an experiment where cornea was digested with a total of 150 ml of pancreatic extract, 6·9 g of polysaccharide was obtained: the amount of carbohydrate due to pancreatic extract being only of the order of a few milligrams.[6]

The use of proteolytic enzymes from plants has been used by several workers. Bromelin, ficin and papain have been used. These enzymes are capable of breaking down tissue proteins rapidly and to small fragments.

There are several techniques, the best of which seems to be that of Scott[7]. He makes use of the fact that papin is a very heat-stable enzyme and thus carries out the digestion at 65 °C. This has two advantages: first no preservative has to be added; secondly the digestion proceeds at a much faster rate.

Before subjecting a tissue to proteolytic digestion it is of importance to prepare the tissue in a suitable way. The tissue should be finely minced or ground and the fats extracted, in order to facilitate the penetration of the enzyme solution into the tissue. A preliminary heat coagulation is of importance in the case of tryptic digestion, since the denatured proteins are more easily attacked by the enzyme.

PURIFICATION OF TISSUE EXTRACTS

After the proteolytic digestion of the tissue the investigator is left with a solution containing amino acids, peptides, salts, fats and nucleic acids or their breakdown products, and in most cases only a few per cent of polysaccharides.

In order to recover the polysaccharides in a reasonable pure form the mixture has to be subjected to some further purification procedure. Most protein and peptide precipitating agents have been used to reach this goal. Unfortunately they have a tendency to precipitate polysaccharides together with the impurities.

A method frequently used for the removal of protein impurities has been the so-called Sevag[8] technique. This method which was originally described by K. A. H. Mörner[9] consists of denaturating the proteins by shaking the solution with chloroform and amyl alcohol.

Schiller *et al.*[4] precipitated the proteins with trichloracetic acid after a combined alkali extraction tryptic digestion procedure. The same precipitant has been used by Balazs[10] after peptic digestion.

The simplest way to dispose of most of the impurities is to precipitate the polysaccharides either by alcohol or by quaternary ammonium compounds.

Alcohol precipitation yields a product which is often largely contaminated with proteins or protein residues, depending on the organ from which the polysaccharide has been isolated. Dialysis to remove split products is not always to be recommended, since, depending on the quality of the dialysis membranes, some losses of polysaccharides may occur. Further purification is frequently required. Protein adsorbants

such as Fullers earth[11] or Lloyds reagent have been used. However, these adsorbents have a tendency to adsorb considerable amounts of carbohydrate-containing material together with proteins.

In our laboratory the following general scheme has been used for the isolation of the polysaccharide (Table 2). Two consecutive precipitations with alcohol, one of alkaline (pH about 9–10), the other of an acid reaction (pH about 2–3), purify the product to a great extent. The extraction of the alcohol-precipitated material with phenol has two advantages and one disadvantage. First the removal of the proteolytic-split products is fairly good and secondly the losses at that stage can be easily demonstrated by precipitating the phenolic solution with alcohol and analysing the precipitate. The disadvantage is that 90% phenol is a very unpleasant solvent to work with, especially on a large scale work.

TABLE 2. PREPARATION OF POLYSACCHARIDES

Freeze-dried organ ⟶ digestion with proteolytic enzymes ⟶
⟶ Precipitation with ethanol at pH 11 ⟶ precipitate dissolved at pH 2 and reprecipitated with ethanol ⟶
⟶ Precipitate dissolved solution neutralized and freeze-dried ⟶
⟶ Extraction with 90% phenol ⟶
⟶ Phenol-insoluble part dissolved in water ⟶ acidified and precipitated with ethanol ⟶
⟶ Dissolved neutralized and precipitated with ethanol (or freeze-dried)

The polysaccharides prepared according to this method generally contain between 5–10 per cent protein as an impurity. This impurity, however, does not interfere with the subsequent fractionation procedure.

Scott[12] has recently described a method for deproteinizing mucopolysaccharide solutions, based on his extensive work on the use of quaternary ammonium compounds for the fractionation of acidic polysaccharides. In this method the solution is treated with a quaternary ammonium compound at an alkaline pH. Both polysaccharides and proteins are thus precipitated. The formation of complexes between polysaccharides and proteins is prevented by the alkaline reaction and by the competition between the quaternary ammonium compound and the proteins. At a faintly acid pH the complexes between the proteins and the quaternary ammonium compound are dissolved, while those of the polysaccharides are not. They can, however, be dissolved by salt solutions from which the polysaccharides can be recovered by alcohol precipitations.

FRACTIONATION OF POLYSACCHARIDE MIXTURES

The most commonly used method for the fractionation of polysaccharide mixture has been the fractional precipitation with organic solvents from aqueous solutions containing different metal ions.

A fractionation scheme, based on the addition of increasing amounts of ethanol to the polysaccharide solution in the presence of calcium ion, has been worked out by Meyer *et al.*[5], and has been used in most of their outstanding work in this field. By their method the known mucopolysaccharides of connective tissue have been separated.

and some of them have also been characterized by their behaviour in the fractionation scheme.

Similar schemes using barium instead of calcium have been used by Smith and Gallop[13] and in our laboratory.[14]

The fractionation with alcohol has certain drawbacks. It is difficult to apply on a microscale, a point of great importance both in metabolic work, and in work where changes of the mucopolysaccharide pattern in diseased organs have to be studied. Moreover the fractions are not always clear cut.

A new approach to the problem was made in 1950 when zone electrophoresis applied to the fractionation of polysaccharides was introduced by Gardell *et al.*[15]. Kieselguhr was used as the supporting medium, and the electrophoresis was performed in an acetate buffer. The supporting medium was packed as a slab which, after completion of the electrophoresis, was cut at right angle to the direction of movement. The polysaccharides were then eluted from each cut and their concentration was determined.

This method was later on used by Boström and Gardell[16] in their work on the metabolism of sulphated mucopolysaccharides in the skin and later on by Dorfman and his group working on the metabolism of the carbon skeletons of these compounds.[4]

Recently electrophoretic separations have been carried out on cellulose columns, which are said to give better results and are easier to handle.[17] For smaller amounts of material Rienits[18] used paper electrophoresis and detected the polysaccharides by means of their metachromatic staining properties.

Fuller and Northcote[19] used silk or glass paper. On these materials other reagents for the detection of polysaccharides like α-naphthol and sulphuric acid can be used, thus allowing polysaccharides other than those with metachromatic properties to be detected.

Paper chromatographic methods have been used by Kerby[20]. However, polysaccharides do not give any discrete spots on paper chromatograms.

Partition analysis, which has made so many important contributions to the analysis of proteins, has also been tried in the separation of mucopolysaccharides. Countercurrent distribution for the separation of commercial heparin samples into several fractions was used by O'Keeffe *et al.*[21] and Wolfrom *et al.*[22] The solvents were amyl alcohol and in order to have the heparin soluble in this solvent aliphatic amines were used as carriers. By using solvents containing aliphatic amines Dorfman and his group succeeded in resolving a mixture of chondroitin sulphuric acid and hyaluronic acid in its components on a column of kieselguhr.[23] The amounts which can be separated are rather small compared to the volumes of solvents necessary and the separations are not always very clear cut.

In 1955 we tried to use chromatography on a cellulose column using the same solvents as were used by Kerby for paper chromatography. It was then found that either the polysaccharide did not move at all on the column or it moved with the solvent front. These experiments led us to work out a method, the principle of which is very simple.[24]

A cellulose column is used and the column is saturated with a solvent which is known to precipitate all polysaccharides in the mixture to be separated. We used 80% ethanol containing 0·3% barium acetate for that purpose. The polysaccharide mixture dissolved in 0·3% barium acetate is then added to the column and the column

is washed with the 80% ethanol 0·3% barium acetate mixture. The polysaccharides are then eluted by using decreasing concentrations of alcohol in the eluting solvent. Similar methods have been used by Desreux and Ghauysen[25] for the separation of nucleic acids and by Zahn and Stahl[26] and Zittle and Della Monica[27] for the separation of proteins.

The possibilities of the method are best illustrated by the following example in which the polysaccharides from nucleus pulposus were fractionated.

From analysis of the unfractionated polysaccharide it was suspected that, besides chondroitin sulphuric acid detected in nucleus pulposus by Malmgren and Sylvén[28], the mixture also contained a sulphated polysaccharide composed of glucosamin, galactose and sulphate thus resembling keratosulphate (Table 3). When fractionated on a cellulose column two main fractions were obtained (Table 4). A continuous gradient was also tested in these fractionation experiments. Two peaks were obtained with widely different properties.

TABLE 3. ANALYSIS OF THE POLYSACCHARIDE MIXTURE OBTAINED FROM NUCLEUS PULPOSUS

	I	II
Nitrogen (%)	3·2	3·3
Ester sulphates (%)	4·9	5·9
Uronic acid (%)	15·7	20·1
Glucosamine (%)	8·8	10·6
Galactosamine (%)	14·8	14·7
Galactose (%)	8·0	12·7
Fucose (%)	—	2·1

TABLE 4. FRACTIONATION OF THE POLYSACCHARIDES FROM NUCLEUS PULPOSUS WITH ALCOHOL AND BARIUM ACETATE ON A CELLULOSE COLUMN

	Fraction eluted with 35% ethanol and 0·3% barium acetate	Fraction eluted with 0·3% barium acetate
Ash (%)	24·3	31·9
Nitrogen	2·6	2·0
Ester sulphates (%)	5·5	4·8
Glucosamine (%)	17·5	0·25
Galactosamine (%)	0·25	18·9
Galactose (%)	16·8	—
Fucose (%)	2·3	—

This is not chromatography in the generally accepted meaning of the term. The cellulose is only to be regarded as a supporting medium on which the polysaccharide is precipitated in a fine layer, and as the composition of the eluting solvent is changed the substances are dissolved and eluted. Thus there is no sorption step and no true adsorption equilibrium.

When Scott[7a] in 1955 made an attempt to improve the chromatographic method of Kerby he found that quaternary ammonium compounds gave insoluble precipitates with acidic polysaccharides.

Stacey[29] simultaneously found that CSA and heparin could be precipitated by cetyltrimethylammonium bromide and Terayama[30] found that cellulose sulphate could also be precipitated with quaternary ammonium compounds.

That polyanions could be precipitated in this way with amines and quaternary amines had been observed earlier. Kuhn[31] found in 1940 that proteins could be precipitated by these compounds and Powney in 1943[32] showed that hexametaphosphoric acid could also be precipitated in a similar way. Also nucleic acids can be precipitated by quatenary ammonium compounds.[33]

Scott found, when working with cetylpyridinum chloride and cetyltrimethyl ammonium bromide and various polysaccharides, that the complexes formed could be dissolved by salts. The salt concentration required varied for one type of amine with the type of polysaccharide. Thus the hyaluronic acid complex needed a concentration of about 0·2 N $MgCl_2$ or KCl to be dissolved. CSA needed about 1 N and heparin needed about 1·2 N KCl or 2 N $MgCl_2$.

Another factor which influences the precipitation is the pH. If the pH is brought to such low values around 2 that the carboxyl groups of hyaluronic acid are not dissociated no complex will be formed.

The properties of the precipitates in salt solutions can be used for the fractionation of acidic polysaccharides in two ways:

(1) The polysaccharides and the quaternary ammonium compound can be mixed in salt solution of such a concentration as to prevent any of the polysaccharides present from being precipitated. By diluting the solution with water the complexes precipitate at their critical salt concentration.

(2) The precipitation of all polyanions in the solution can be accomplished in very dilute salt concentration and the precipitate is then extracted with salt solutions of increasing concentrations.

When working with a batch-wise procedure the first principle is the best. Scott *et al.*[34] used this method for the fractionation of heparin, and Ricketts[35] used the principle in the fractionation of dextran sulphate. It has also been used by Schiller[36] in the fractionation of heparin monosulphate.

A great many complications have to be dealt with if the second principle is used. The precipitate has to be divided into small particles and the extraction has to be continued for a long period of time. And the possibility still exists that more soluble particles are trapped in particles consisting of less soluble material.

However, this principle can be used in a column procedure similar to that used for alcohol fractionation. In this case the cellulose column is saturated with the quaternary ammonium compound. An aqueous solution of the polysaccharides is introduced at the top of the column and allowed to drain into it. The column is then washed with a fresh portion of the precipitating solvent. By then eluting the column by solvents of increasing salt concentrations the polysaccharides are eluted (Fig. 1).[37]

Although neutral polysaccharides do not form complexes with CPC and CTAB at neutrality they can form complexes if the pH is brought to about 12, where the hydroxyl groups are dissociated. In this case only CTAB can be used since CPC is decomposed at alkaline reaction.[38, 39] Another possibility is to add borate and the resulting acid, polysaccharide can then form a precipitate with a quaternary ammonium compound.[38,39]

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Recently a new principle has been used in the fractionation of acidic polysaccharides. The modified cellulose ion exchange materials introduced by Sober and Peterson[40] for fractionation of proteins have been used by Ringertz and Reichard[41, 42] for separation of several mucopolysaccharides.

The separation is carried out on *Ecteola*[43] at an acid reaction, i.e. pH about 2 with increasing concentrations of sodium chloride.

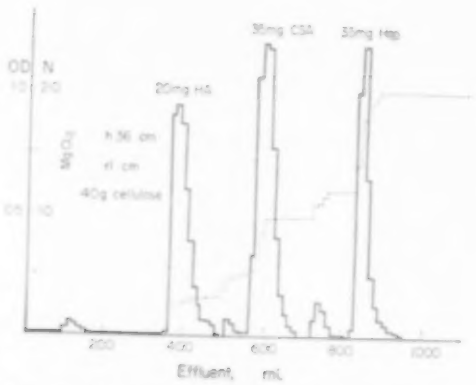

FIG. 1. Separation of polysaccharides on a cellulose column by means of cetylpyridinium chloride and magnesium chloride (Borelius, Gardell and Scott[37]).

It has for many years been suggested that changes in the polysaccharide composition of connective tissue takes place in different diseases. However, such changes have only been demonstrated in very few cases. It is very likely that with the recent developments in technique a new approach to these questions can be made with greater success particularly if the methods can be applied on a microscale.

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ON THE METABOLISM OF MUCOPOLYSACCHARIDES

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Abstract—Aspects of the biosynthesis of mucopolysaccharides are discussed. Substances dealt with include the hexosamine-containing compounds which occur in mammalian tissues; these are hyaluronic acid, chondroitin and the chondroitin sulphates, heparin, heparitin sulphate and keratosulphate. Mechanisms of synthesis of the mucopolysaccharides have been derived from the use of radioactive precursors such as ^{14}C-labelled glucose or ^{35}S-labelled sulphate and by knowledge of the enzymatic reactions of the various constituents of the compounds. Experimental work concerning these two phases is discussed in some detail. Finally evidence is given of polysaccharide or macromolecule formation.

While much of our knowledge about the chemistry of the acid mucopolysaccharides dates back to the nineteenth century, the current concepts about the metabolism of these substances have resulted mainly from the work of the past decade. However, in 1861, one of the earliest workers in the field of connective tissue chemistry, Boedeker, also contributed the first known metabolic experiment.[1] Boedeker, who studied the carbohydrate-containing material of cartilage, administered a cartilage jelly from autopsy material to volunteers and observed an increased excretion of sulphate in the urine.

The introduction of the isotope technique, in conjunction with the development of methods for small-scale isolation of mucopolysaccharides and their components, has increased enormously the possibilities of carrying out metabolic studies in the mucopolysaccharide field. There exists today a vast literature on the metabolism of mucopolysaccharides summarized in a number of recent reviews,[2–9] and this paper will be confined to a few aspects of the biosynthesis of these compounds. The group of substances, which will be dealt with, includes the following hexosamine-containing polysaccharides, which occur in mammalian tissues: hyaluronic acid, chondroitin, the three different chondroitin sulphates, heparin, heparitin sulphate and keratosulphate. Table 1 gives a survey of the compounds under discussion and of their components.

The first enzymic polysaccharide synthesis was the well-known phosphorylase reaction catalysing the formation of glycogen from glucose-1-phosphate.[10] Each successive step in the formation of glycogen involves the addition of a glucose residue from glucose-1-phosphate. In the case of the mucopolysaccharides, which belong to the group of heteropolysaccharides composed of dissimilar units, we are faced with the following problems: (1) Which are the precursors of the different constituents and how are they formed? (2) How is the macromolecule formed from its precursors with the concomitant alternation of the various units? With regard to the first of these

questions, a good deal of knowledge has now accumulated, whereas the answer to the second question is still completely unknown.

The study of mucopolysaccharide biosynthesis has derived great benefit from the application of results gained in other fields of carbohydrate metabolism and, especially, numerous examples of the participation of various uridine compounds in saccharide synthesis makes it likely that such substances are the specific precursors of the mucopolysaccharides as well. Some instances of polysaccharide synthesis have already

TABLE 1. CONSTITUENTS OF ACID MUCOPOLYSACCHARIDES

Name	Hexosamine	Uronic acid	Galactose	Sulphate
Hyaluronic acid	Acetylglucosamine	Glucuronic acid	–	–
Chondroitin	Acetylgalactosamine	Glucuronic acid	–	–
Chondroitin sulphate A	Acetylgalactosamine	Glucuronic acid	–	+
Chondroitin sulphate B	Acetylgalactosamine	Iduronic acid	–	+
Chondroitin sulphate C	Acetylgalactosamine	Glucuronic acid	–	+
Heparitin sulphate	Acetylglucosamine	Glucuronic acid	–	+
Heparin	Glucosamine	Glucuronic acid	–	+
Keratosulphate	Acetylglucosamine	–	+	+

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been reported, where the immediate precursor is a uridine nucleotide, namely, glycogen,[11] cellulose,[12] chitin[13] and hyaluronic acid.[14, 15] In fact, it seems possible that the uridine diphosphate glucose–glycogen *trans*glucosylase reaction may represent the normal route of glycogen synthesis, while the phosphorylase reaction, though reversible, is presumably responsible mainly for the degradation to glucose-1-phosphate.

Theories about the synthesis of the mucopolysaccharides today rest on a composite basis which is formed partly of knowledge about the enzymic reactions of the various constituents of the mucopolysaccharides, partly of studies using radioactive precursors like ^{14}C-labelled glucose or ^{35}S-labelled sulphate. Furthermore, the mere existence of certain compounds such as uridine diphosphate galactosamine sulphate[16] seems to indicate that they may be involved in mucopolysaccharide formation. Finally, there is a smaller amount of knowledge more directly concerned with the formation of the macromolecule.

ENZYMIC FORMATION OF MONOSACCHARIDE MOIETIES

The synthesis of mucopolysaccharides from glucose has for a long time been considered to follow a pathway, in which uridine nucleotides represent the final stage before polymerization to a macromolecule. This has so far been proven only in two instances, i.e. the formation of chitin[13] from uridine diphosphate acetylglucosamine and of hyaluronic acid[14, 15] from uridine diphosphate acetylglucosamine and uridine diphosphate glucuronic acid. Several uridine nucleotides exist, however, which are likely precursors in the biosynthesis of mucopolysaccharides, and the following discussion of the enzymic formation of the monosaccharide moieties will therefore be carried through the enzymic synthesis of these various compounds.

The characteristic constituent of the mucopolysaccharides is hexosamine, which may be either glucosamine or galactosamine. In addition, a uronic acid is present in

the substances under discussion except for keratosulphate, which contains galactose instead. In some of the mucopolysaccharides, which are sulphated, the introduction of the sulphate group into the molecule presents a special problem implying the activation of the sulphate group to adenosine 3′-phosphate-5′-phosphosulphate[17] and the subsequent transfer of the sulphate group to a carbohydrate acceptor. The question of the sulphate activation has been discussed at length in another symposium at this meeting[18] and will therefore be touched on only briefly.

Hexosamine synthesis

The first evidence concerning the enzymic formation of hexosamine was obtained by Leloir and Cardini.[19] From the chitin-producing mould *Neurospora crassa* they prepared an enzyme system, which catalysed the synthesis of glucosamine-6-phosphate from glutamine and glucose-6-phosphate—the key intermediate of glucose metabolism. Subsequent studies of this reaction by Blumenthal *et al.*[20] showed that fructose-6-phosphate was in fact the substrate for the amination. An enzyme catalysing the same reaction has also been isolated from liver by Pogell and Gryder[21]. Though Pogell maintained that glucose-6-phosphate is the substrate of the mammalian enzyme rather than fructose-6-phosphate, it has been claimed by Roseman *et al.*[22] that fructose-6-phosphate is also the substrate for the liver enzyme. In extracts from cartilage formation of glucosamine-6-phosphate from glucose-6-phosphate and glutamine could also be demonstrated.[23]

An alternate pathway for the hexosamine formation exists, in which ammonia substitutes for glutamine as the nitrogen donor, while fructose-6-phosphate, even in this reaction, is the hexose component.[24, 25] In contrast to the equilibrium conditions prevailing in the glutamine reaction,[19] which favour hexosamine synthesis, the equilibrium of the ammonia reaction is displaced towards fructose-6-phosphate and ammonia. This does not, however, necessitate the conclusion that under physiologic conditions the latter reaction does not operate in the direction of hexosamine synthesis. On the contrary, it has been suggested[25] that the acetylation of the reaction product, glucosamine-6-phosphate, *in vivo* pulls the reaction towards the formation of the amino sugar phosphates. The enzyme catalysing this reaction, glucosamine-6-phosphate acetylase, was first isolated from yeast,[26] but it has also been found in several animal tissues.[27]

Other routes leading to the formation of hexosamine-6-phosphates can be considered, i.e. the phosphorylation of glucosamine[28–31] or N-acetylhexosamine,[28, 32–34] yielding glucosamine-6-phosphate and N-acetylglucosamine-6-phosphate, respectively. The former reaction is catalysed by the non-specific glucokinase, while the latter requires a specific enzyme. Under *in vivo* conditions, however, these reactions are probably not operating to a large extent, an assumption which is supported by the fact that both free and acetylated hexosamines are absent from tissues and body fluids except possibly in one instance, namely, cerebrospinal fluid.[35]

In the next step toward a hexosamine-containing uridine nucleotide, N-acetylglucosamine-6-phosphate is transformed to N-acetylglucosamine-1-phosphate in a reaction, which is catalysed by an enzyme similar to phosphoglucomutase.[36] A partial separation of the phosphoglucomutase and phosphoacetylglucosamine mutase activities has been obtained, indicating that the two reactions are catalysed by different enzymes.

Finally, in complete analogy with the reactions incorporating glucose into uridine diphosphate glucose, N-acetylglucosamine-1-phosphate reacts with uridine triphosphate yielding uridine diphosphate acetylglucosamine.[37–39] In addition to its participation in the synthesis of chitin and hyaluronic acid, uridine diphosphate acetylglucosamine can also be expected to be the precursor of the N-acetylglucosamine moieties of keratosulphate and heparitin sulphate. Besides N-acetylated glucosamine residues, the latter substance also contains N-sulphated glucosamine.[40] Though the precursor of this non-acetylated glucosamine moiety is not known, it seems likely that its synthesis occurs via uridine diphosphate glucosamine, the formation of which has been demonstrated in yeast.[38] This substance could likewise be expected to be a heparin precursor, as most of the amino groups of heparin are also sulphated.

Concerning the biosynthesis of the galactosamine moiety of chondroitin and the three chondroitin sulphates nothing is known, but quite recently the normal pathway for its formation seems to have been found by the discovery of an enzyme which catalyses the reaction:

uridine diphosphate acetylglucosamine
↓
uridine diphosphate acetylgalactosamine[41, 42]

This enzyme effects a change similar to the conversion of uridine diphosphate glucose to uridine diphosphate galactose, and thus, a close analogy has again been established between the transformations of the neutral hexoses and those of the hexosamines.

Uronic acid

Several uronic acids are now known to occur in nature. In the acid mucopolysaccharides the uronic acid has always been found to be D-glucuronic acid except in one instance, chondroitin sulphate B, which contains L-iduronic acid—the 5-epimer of D-glucuronic acid. So far, no enzymic reactions concerned with the formation of L-iduronic acid have been reported.

The biosynthesis of glucuronides has been the subject of investigation since the days of Fischer, when the formation of conjugated glucuronic acid was considered to proceed through a glucoside, which was subsequently oxidized to a uronic acid[43, 44]. Later work with tissue slices[45] seemed to indicate a synthesis via three-carbon metabolites, and *in vivo* experiments with ^{14}C-labelled precursors (lactate, pyruvate, glycerol and glucose),[46, 47] which were administered together with glucuronidogenic substances, pointed in the same direction.

Results at variance with this conclusion were obtained, however, in other experiments utilizing glucose-1-^{14}C and glucose-6-^{14}C,[48–50] in which incorporation of the glucose without rearrangement of the chain occurred. The problem was finally solved by the isolation of a thermostable factor involved in glucuronide formation in cell-free liver suspensions, which was subsequently identified as uridine diphosphate glucuronic acid.[51–54] The original hypothesis of Fischer and Piloty was subsequently shown to contain a nucleus of truth by the demonstration of Strominger *et al.*[55] that uridine diphosphate glucuronic acid is formed by way of oxidation of uridine diphosphate glucose by a diphosphopyridine nucleotide-dependent enzyme, uridine diphosphate glucose dehydrogenase. It is obvious that uridine diphosphate glucuronic acid

is a likely precursor of the glucuronic acid moiety of mucopolysaccharides, and its participation in streptococcal hyaluronic acid synthesis has been demonstrated.[15]

ISOTOPIC STUDIES

It is natural that the introduction of isotope methods, which have revolutionized the whole field of metabolic studies, has also proved to be very fruitful in the elucidation of mucopolysaccharide metabolism. The isotope methods have been used mainly for two purposes. First, the origin of the different moieties of the mucopolysaccharides has been investigated in bacteria and in mammals by using precursors such as ^{35}S-sulphate, ^{14}C-acetate and variously labelled ^{14}C-glucose. Secondly, the turnover of the mucopolysaccharides has been studied in normal animals as well as under the influence of various endocrine agents. The first studies of this kind were made by Dziewiatkowski *et al.*[56], who studied the ^{35}S-fixation in cartilage *in vivo*, and by Layton and Frankel[57] who observed on ^{35}S-fixation *in vitro*. This work was later extended by Dziewiatkowski[58] and Boström[59], who demonstrated that the ^{35}S-incorporation into cartilage occurred in the sulphate group of chondroitin sulphuric acid. Later on, studies on the carbon skeleton[60–62] established that the glucose molecule is incorporated without scission of the chain into hyaluronic acid of streptococci in such a way that C(1) of the glucose becomes C(1) in glucosamine as well as in glucuronic acid. Similar results pertain to the formation of hyaluronic acid of animal tissues.[63]

Tracer methods thus may give valuable hints about possible reaction sequences in the biosynthetic pathways, and this type of work should be regarded as preliminary to a closer investigation of the enzymic reactions involved. So far the pilot investigations on the origin of the carbon skeleton carried out with the isotope methods have generally been confirmed by later enzymic studies. In the case of L-iduronic acid such a pilot study is the only investigation so far of the origin of this substance, for which no enzymic reactions are known.[63] Even in this case, the glucose molecule is incorporated without cleavage into the L-iduronic acid of chondroitin sulphuric acid B.

In addition to the usefulness in the studies of mucopolysaccharide precursors, the isotopic methods are indispensable in providing information concerning the turnover of the different mucopolysaccharides *in vivo*. The first studies of this kind were made by Boström in 1952 on costal cartilage of rats.[59] A large number of rats were given ^{35}S-sulphate, and at different times after the injection a number of animals were killed and the radioactivity of various sulphur fractions measured. The labelling of inorganic sulphate decreased very rapidly. The chondroitin sulphuric acid curve had, however, another shape and from the descending part of this curve the biological half-life was found to be be around 16 days. In a similar study on chondroitin sulphuric acid prepared from skin, Boström and Gardell found a half-life of chondroitin sulphuric acid in this tissue of 9–10 days.[64]

The knowledge gained from the ^{35}S studies was subsequently confirmed by Schiller *et al.* using ^{14}C-labelled glucose and acetate to study the turnover of skin mucopolysaccharides.[65, 66] The outcome of these studies was the demonstration of the similar turnover rates of the different moieties of the chondroitin sulphuric acid molecule, the sulphate, hexosamine, glucuronic acid and acetyl groups, which indicates that the whole molecule turns over as an entity. The use of ^{14}C-labelled precursors also made it

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possible to study the turnover of the hyaluronic acid, which contains no sulphate. This mucopolysaccharide showed a particularly rapid turnover rate with a half-life time of approximately 2–3 days.

While *in vivo* studies utilizing labelled precursors may give valuable clues to the existence of specific enzymic steps, it is also sometimes possible to corroborate by the isotopic methods the existence *in vivo* of an enzymic reaction demonstrated *in vitro* and to estimate its quantitative significance. In the era of enzymology it seems justified to keep in mind that what we really want to know is what is going on within the living, unbroken tissue.

As our group has made extensive studies of the metabolism of slices of cartilage especially utilizing ^{35}S-labelled sulphate, we would like to exemplify the possibilities of the isotopic methods from our own experience. There are several advantages of the ^{35}S technique over ^{14}C methods among which can be mentioned the following:

(1) The low isotope costs, amounting to only 1 per cent or less of those of suitable ^{14}C precursors.

(2) The specificity of ^{35}S methods due to the very limited incorporation of the isotope into compounds other than sulphate esters.

(3) The high specific activity of the isotope, which gives the ^{35}S methods a very high sensitivity.

The *in vitro* system in question was developed by Boström in collaboration with Månsson in 1952.[67] Working with this system Boström and Månsson found in 1953 that the addition of a liver homogenate greatly increased the incorporation of ^{35}S.[68] After some laborious purification work we were able to crystallize the factor responsible for this effect and prove its identity with glutamine.[69, 70] It so happened that one of our best purification steps was a fractional precipitation with mercuric salt, which was essentially the same method as that used 75 years ago by Schulze and Bosshard, in the first isolation of glutamine.[71]

The identity of the liver factor with glutamine clearly indicated that the effect on ^{35}S incorporation did not primarily involve the process of sulphate esterification as the amide group of glutamine had been shown to be the precursor for hexosamine nitrogen in two microbial systems. The first demonstration of a role for glutamine in hexosamine synthesis was the finding of Leloir and Cardini that an extract of *Neurospora crassa* could synthesize glucosamine-6-phosphate from glucose-6-phosphate and glutamine.[19] Subsequently Lowther and Rogers showed that glutamine was a precursor in the formation of hyaluronic acid in a resting cells suspension of streptococci.[72, 73] These findings actually led us on the right track in the isolation of the liver factor stimulating the incorporation of ^{35}S in chondroitin sulphate. Evidence that glutamine was not specifically involved in the sulphate esterification was obtained by studying its influence on the formation of other parts of the chondroitin sulphate molecule as well, and it was also found that the incorporation of glucose-^{14}C and acetate-^{14}C was enhanced by glutamine.[74] In addition, the incorporation of glucose-^{14}C into the galactosamine moiety of chondroitin sulphate was increased much more than the increase in labelling of the uronic acid moiety. Furthermore, glucosamine stimulated the ^{35}S uptake to approximately the same extent as glutamine.[75] The simultaneous presence of the two substances did not result in a further increase in ^{35}S uptake. Therefore, it seems justified to conclude that the observed effects of

glutamine on the cartilage system can be fully explained by assuming that glutamine participates in the synthesis of the galactosamine moiety of the chondroitin sulphate. As was pointed out previously two reactions have been described, which use glutamine and ammonia, respectively, as nitrogen donors for the hexosamine formation.[19, 24, 25] On the basis of our results we believe that the glutamine reaction is the one used by the cartilage. This assumption is further strengthened by the fact that the decline in the

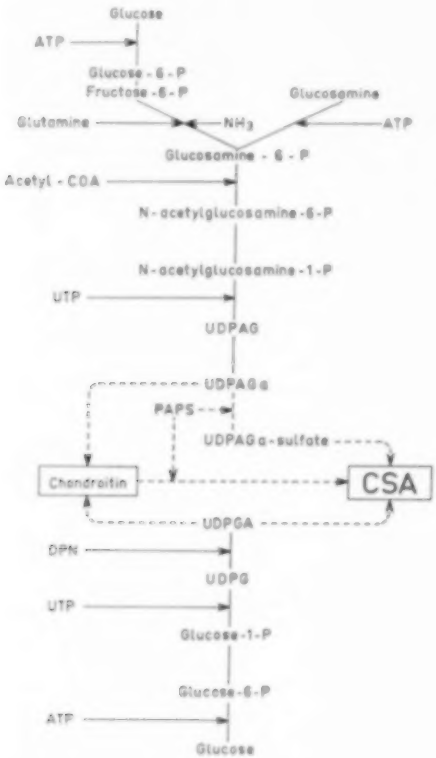

FIG. 1 Biosynthesis of CSA. Possible pathways

Abbreviations used: P for phosphate; ATP for adenosine triphosphate; Acetyl-CoA for acetyl-coenzyme A; UTP for uridine triphosphate; UDPAG for uridine diphosphate acetylglucosamine; PAPS for adenosine 3′-phosphate-5′-phosphosulphate; CSA for chondroitin sulphate; UDPGA for uridine disphosphate glucuronic acid; DPN for diphosphopyridine nucleotide and UDPG for uridine diphosphate glucose.

rate of ^{35}S incorporation with time can be abolished by the addition of glutamine to give a straight line for several hours.[74]

POSSIBLE INTERMEDIATES IN POLYSACCHARIDE FORMATION

Some indirect evidence has also been brought forward in the discussion of different possible pathways of biosynthesis of the mucopolysaccharides (Fig. 1). In particular, the mere existence of certain compounds seems to indicate the occurrence of various

enzymic reactions, which could possibly participate in the formation of a mucopolysaccharide molecule. One such compound is the galactosamine and sulphate-containing uridine nucleotide of Strominger[16], uridine diphosphate acetylgalactosamine sulphate. It is very tempting to assume that this substance is a metabolite on the main pathway in the synthesis of chondroitin sulphate. On the other hand, Meyer *et al.*[76] have isolated polysaccharide fractions from the cornea with a sulphur content that varies between zero and 1 mole of sulphate per disaccharide unit. This fact may indicate that the sulphation occurs after the polymerization of the molecule and not on the monomer stage. Another compound also isolated by Strominger[16] is uridine diphosphate acetylglucosamine-6-phosphate, a compound for which no obvious function yet can be conceived.

POLYSACCHARIDE FORMATION

Turning to the formation of the macromolecules this is a field which has only recently been opened up. It was early suggested by Leloir[19] that uridine diphosphate acetylglucosamine could possibly participate in the formation of chitin. Storey and Dutton[53] likewise suggested that uridine diphosphate glucuronic acid was a precursor of uronic acid-containing mucopolysaccharides. These hypotheses are now being substantiated by the demonstration of the participation of uridine nucleotides in the formation of several polysaccharides. The first well-documented example of the participation of a uridine nucleotide in polysaccharide synthesis is the demonstration of chitin synthesis from ^{14}C-labelled uridine diphosphate acetylglucosamine in a cell-free extract of *Neurospora crassa*.[13] The need of a primer in this reaction in the form of short-chain chitodextrins was also demonstrated. A similar study was also made on the formation of cellulose in an extract of *Acetobacter xylinum* using uridine diphosphate glucose as a substrate and cellodextrin as primer.[12]

Three years ago Leloir and Cardini[11] showed that a liver enzyme can catalyse a net synthesis of glycogen from uridine diphosphate glucose in the presence of primer glycogen or soluble starch. It was suggested that the essentially irreversible uridine diphosphate glucose reaction is used for the synthesis of glycogen, while the reversible phosphorylase reaction should act on the degrading side of glycogen metabolism.

Concerning the mucopolysaccharides under discussion information is more scarce. In 1955, Glaser and Brown observed incorporation of label into hyaluronic acid in an extract of Rous chicken sarcoma using acetyl-labelled uridine diphosphate acetylglucosamine and unlabelled uridine diphosphate glucuronic acid as precursors, though considerable loss of label occurred during purification of the hyaluronic acid.[14]

A more potent preparation has been obtained by Markovitz *et al.*[15] from streptococci. The precursors used were labelled with tritium using the elegant Wilzbach technique which consists simply of incubating a non-radioactive sample of the substance to be labelled with tritium gas followed by repurification.[77] In this way incorporation of tritium-labelled uridine diphosphate glucuronic acid, uridine diphosphate acetylglucosamine and acetylglucosamine-1-phosphate into hyaluronic acid could be achieved.

Concerning the formation of the other non-sulphated polysaccharide, chondroitin, nothing is known.

The formation of the sulphated mucopolysaccharides poses the special problem of the sulphate acceptor, and some progress has been made in this respect. The first

demonstration of the synthesis of a sulphated polysaccharide in a cell-free system was D'Abramo's and Lipmann's demonstration of an incorporation of ^{35}S-sulphate and ^{35}S-labelled "active sulphate", adenosine 3'-phosphate-5'-phosphosulphate, into chondroitin sulphate in an extract of embryonic cartilage.[78] This work gave no information, however, about the possible site of the sulphation. In subsequent work by Suzuki and Strominger[79] it could be shown that a soluble extract of oviduct was able to catalyse the incorporation of label from ^{35}S-labelled "active sulphate" into four sulphated polysaccharides including the three different chondroitin sulphates. The presence of primer was necessary for the reaction. Continued work along these lines by Suzuki[80] has shown that a transfer of sulphate can be obtained to certain oligosaccharides derived from chondroitin sulphate by enzymatic hydrolysis.

Recent work by Korn[81] and by Spolter and Marx[82] on the formation of heparin in cell-free systems from mouse mastocytomas seems to indicate that sulphation can occur on the polymer stage. The same view is also favoured by the work of Greiling and Bauditz[83], who showed that chondroitin sulphates and desulphated chondroitin sulphates could serve as sulphate acceptors in particle-free extracts prepared from liver. Still more conclusive in this respect are the experiments preliminarily reported by Delbrück and Lipmann[84] who showed that a purified enzyme preparation from embryonic cartilage, obviously free from carbohydrate precursors, could transfer sulphate from "active sulphate" to the three chondroitin sulphates or to chondroitin but not to hyaluronic acid, heparin or keratosulphate.

Concerning the biosynthesis of the carbon skeleton of the sulphated polysaccharides in cell-free systems nothing is known as yet, but the existence of several such systems makes it probable that these secrets are also beginning to be unravelled.

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Papers to be published in future publications

ROBERT C. WOOD, ROBERT FERONE and GEORGE H. HITCHINGS: The relationship of cellular permeability to the degree of inhibition by amethopterin and pyrimethamine in several species of bacteria.

KENNETH E. MOORE and THEODORE M. BRODY: The effect of triethyltin on oxidative phosphorylation and mitochondrial adenosine triphosphatase activation.

KENNETH E. MOORE and THEODORE M. BRODY: The effect of triethyltin on mitochondrial swelling.

KARL A. SCHELLENBERG and G. ROBERT COATNEY: The influence of antimalarial drugs on nucleic acid synthesis in *Plasmodium gallinaceum* and *Plasmodium berghei*.

E. D. WILLS: Effect of unsaturated fatty acids and their peroxides on enzymes.

PAUL BIJVOET and HARRIS BUSCH: Effect of 5-bis (2-chlorethyl) aminouracil on incorporation of uracil-2-^{14}C into RNA of tumour-bearing rats.

A. CHARI-BITRON: A study of the toxicity and the biochemical effects of monofluoromalonate.

HENRIK WALLGREN: Effects of acetylcholine analogues and ethanol on the resaration of brain cortex tissue *in vitro*.

W. N. ALDRIDGE and J. M. BARNES: Neurotoxic and biochemical properties of some tri-aryl phosphates.

J. J. ROBERTS and G. P. WARWICK: The mode of action of alkylating agents—II. Studies of the metabolism of myleran. The reaction of myleran with some naturally occurring thiols *in vitro*.

J. J. ROBERTS and G. P. WARWICK: The mode of action of alkylating agents—III. The formation of 3-hydroxytetrahydrothiophene-1:1-dioxide from 1:4-dimethylsulphonyloxybutane (Myleran), 5-β-Lalanyltetrahydrothiophenium mesylate, tetrahydrothiophene and tetrahydrothiophene-1:1 dioxide in the rat, rabbit and mouse.

D. M. SERRONE and J. M. FUJIMOTO: Inhibition of the metabolism of hexobarbital *in vitro*.

J. BELFORD and MARCIA R. FEINLEIB: Phosphorylase activity in heart and brain after reserpine iproniazid and other drugs affecting the central nervous system.

N. E. JONGEJANS-SICKLER, A. KEMP and J. VAN NOORDWIJK: Evaluation of some uncoupling agents in the chemotherapy of experimental cancer: A study of their distribution in tumour-bearing rats.

JILL E. CREMER: A comparison of the action of triethyltin with other drugs on creatin phosphate levels in rat brain and diaphragm preparations.

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THE RELATIONSHIP OF CELLULAR PERMEABILITY TO THE DEGREE OF INHIBITION BY AMETHOPTERIN AND PYRIMETHAMINE IN SEVERAL SPECIES OF BACTERIA

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Abstract—As a possible means for determining the influence of cellular permeability on the action of the antifolic acid drugs in bacteria, a comparison has been made of their effects on whole cells and on cell-free extracts of several species. The parameters examined were inhibition of growth, inhibition of folinic acid synthesis in whole cells and inhibition of folinic acid synthesis in cell-free extracts. In *Streptococcus faecalis* the concentrations of amethopterin and pyrimethamine required to inhibit all of these functions are in the order of several millimicrograms per milliliter. In *Escherichia coli* and to a more limited extent in *Lactobacillus arabinosus*, millimicrogram amounts of these drugs will inhibit the synthesis of folinic acid by the extracts, whereas microgram concentrations are required to produce the inhibitions in the whole cells. These results suggest that certain non-folic acid-requiring bacteria such as *E. coli* and *L. arabinosus* are relatively insensitive to the antifolic acid drugs because they apparently are unable to assimilate them with as much facility as are the folic acid-requiring bacteria such as *S. faecalis*. It does not appear that the intracellular enzymes that convert folic acid to folinic acid in the non-exacting bacteria are insensitive to the drugs.

The following results suggest that the uptake by *L. arabinosus* of amethopterin (or aminopterin), but not that of folic acid, is mediated by an active cellular transport system which normally takes up thiamin.

(1) The inhibition of growth produced by amethopterin can be prevented by thiamin or by thiamin plus pyridoxal, but not by comparable concentrations of folic or folinic acids.

(2) In washed-cell suspensions thiamin interferes with the uptake of aminopterin, but not of folic acid. Glucose stimulates the uptake of aminopterin and of folic acid.

(3) In washed-cell suspensions thiamin interferes with the inhibitory action of amethopterin on the synthesis of folinic acid.

For the following reasons the uptake of pyrimethamine by *L. arabinosus* seems to be of a passive, diffusive nature, not involving a thiamin permease system.

(1) The inhibition of growth produced by pyrimethamine cannot be reversed by thiamin or by thiamin plus pyridoxal or by folic or folinic acids.

(2) Thiamin has no effect on the uptake of pyrimethamine by washed-cell suspensions. Glucose inhibits the uptake of pyrimethamine. The drug is taken up equally well by living or dead cells. Its uptake is dependent upon the pH of the system, more drug being absorbed at values closer to its pK_a.

(3) In washed-cell suspensions pyrimethamine inhibits the conversion of folic acid to folinic acid, but this inhibition is not counteracted by thiamin.

The possible exploitation of the high degree of specificity of certain permease systems for the purposes of practical chemotherapy is discussed.

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INTRODUCTION

The work of McGlohon *et al.*[1] and of McGlohon and Bird[2] demonstrated that the growth of certain non-folic acid-requiring micro-organisms, including *Lactobacillus arabinosus*, is inhibited by rather low concentrations of the anti-folic acid drugs, and that this inhibition could not be counteracted by reasonable levels of folic or folinic acids. Since Hendlin *et al.*[3] have shown that the anti-folic acid drugs block the synthesis of folinic acid in *L. arabinosus*, the inability of this factor to counteract growth inhibition by these drugs in *L. arabinosus* seemed anomalous. Studies on the uptake of folic acid, leucovorin, and the anti-folic acid drugs by bacteria[4] have indicated that there is a high degree of specificity with regard to the comparative efficiency with which these compounds are assimilated by various species. For example, *Pediococcus cerevisiae* assimilates leucovorin with ease, but it is unable to take up folic acid unless the latter is supplied at 1000 times the concentration of leucovorin.[5] It seemed possible that leucovorin may be unable to counteract the effects of the anti-folic acid drugs in *L. arabinosus* and in similar organisms because it may be only poorly assimilated by the cells. The following studies resulted from this suggestion and deal with the concentrations of amethopterin and pyrimethamine* required to inhibit growth and folinic acid synthesis by intact cells as compared to cell-free extracts. In addition, the means by which these drugs permeate cells of *L. arabinosus* has been investigated in more detail.

There is a very strict specificity with regard to the utilization of folic acid and its derivatives. Certain micro-organisms which require *p*-aminobenzoic acid for growth are unable to utilize *p*-aminobenzoylglutamic, folic or folinic acids, even though these compounds are end-products of *p*-aminobenzoic acid metabolism. Examples of these organisms are strains of *Clostridium acetobutylicum*,[6] *Acetobacter suboxydans*,[6] *Escherichia coli*,[4, 7–9] *Neurospora crassa*.[10] The inability of bacteria such as certain *E. coli* mutants to utilize folic acid for growth in place of *p*-aminobenzoic acid is paralleled by the finding that this species can incorporate *p*-aminobenzoic acid–carboxyl-^{14}C,[11, 12] but not folic acid-2-^{14}C.[12] *P.cerevisiae* cannot utilize reasonable concentrations of folic acid for growth in place of leucovorin,[13] even though it can convert folic acid to folinic acid.[5, 5] However, it can utilize *high* concentrations of folic acid for growth[13, 14] and can be trained to grow on lower concentrations of folic acid.[14] This training is accompanied by a greatly increased sensitivity to aminopterin and amethopterin.[14]

Folic acid and its deratives have been found to be incapable of reversing the growth inhibitory effects of the sulfonamides in a number of micro-organisms. This is despite the fact that the sulfonamides have been clearly shown to interfere with the synthesis of folic acid.[9] Examples of species in which folic acid is incapable of reversing sulfonamide bacteriostasis are *E. coli*,[9, 15–19] *Aerobacter aerogenes*,[16] *Staphylococcus aureus*,[18] and *Diplococcus pneumoniae*.[18] Presumably *p*-aminobenzoic acid could counteract the inhibition by sulfonamides in all of these species. In *Mycobacterium tuberculosis* folic acid was incapable of counteracting the inhibition of growth produced by *p*-aminosalicylic acid, whereas *p*-aminobenzoic acid did effect a reversal.[20] Moreover, folic or folinic acids or their derivatives were unable to counteract the action of the anti-folic acid drugs in *Bacillus subtilis*,[21] *N. crassa*,[22] *Streptococcus pyogenes*,[2] or *E. coli*.[23]

* Pyrimethamine ("Daraprim") is 2:4-diamino-5-*p*-chlorophenyl-6-ethylpyrimidine.

Concentrations of the anti-folic acid drugs which are many times higher than those required to inhibit *Streptococcus faecalis* or *Lactobacillus casei* have been found necessary to inhibit the growth of non-folic acid-requiring organisms such as *E. coli*,[11, 23–27] *A. aerogenes*,[28] *Lactobacillus plantarum*,[25] *N. crassa*,[22] and *Schizosaccharomyces octosporus*,[25] despite the fact that these micro-organisms presumably convert folic acid to folinic acid.

These seemingly contradictory observations made by earlier investigators in the folic acid field, who used intact bacterial cells, could be explained on the basis of permeability differences among the various bacterial species tested. It is the purpose of this paper to report observations which may explain many of the apparent discrepancies.

METHODS

Bacterial strains employed and their cultivation

Streptococcus faecalis (ATCC 8043) was grown in Difco folic acid-assay medium containing either folic acid (1 mμg/ml) or leucovorin, as indicated in the tables *Lactobacillus arabinosus* 17–5 (ATCC 8014) was grown in the semi-synthetic medium of Shiota.[29] "Salts B", included but not described by him, were considered to be anhydrous $MgSO_4$ (100 mg) and 10 mg each of NaCl, $MnSO_4$ and $FeSO_4 \cdot 7H_2O$ per liter of single-strength medium. The complete medium contained *p*-aminobenzoic acid, thiamin, and pyridoxal at 10 mμg, 1 μg and 2μg per ml, respectively. *Escherichia coli* M48–34 (originally from Dr. B. D. Davis) was grown in medium "A" of Lascelles and Woods[9]. Ten milligrams of Na_2HPO_4 per ml were added to this medium in order to bring the pH to 7·2 without further adjustment. To supply the *p*-aminobenzoic acid requirement of this strain of *E. coli*, 5 mμg of the vitamin were added per ml of medium. *Bacillus subtilis* (Merck 3R 8788) was grown in a medium originally described for the growth of *Proteus vulgaris*.[30] Growth was measured turbidimetrically using the Klett–Summerson photoelectric colorimeter with filter no. 66.

Folic acid to folinic acid reaction systems

The preparation of these systems from cells and extracts of *S. faecalis* has been described.[31] Washed-cell suspensions of *L. arabinosus* were prepared from cells harvested from the medium described above. Experience showed, however, that it was necessary to grow the cells in a limiting amount of *p*-aminobenzoic acid (i.e. 0·2 mμg/ml) in order to achieve appreciable synthesis of folinic acid. The details of the reaction systems are given in the tables. *E. coli* M48–34 was also cultured in a limiting concentration of *p*-aminobenzoic acid (less than 6 mμg/ml). Maximum synthesis of folinic acid by washed cells of *E. coli* occurred in the presence of L-glutamic acid; less synthesis occurred with sodium formate, and practically none occurred when both of these substrates were present.

Cell-free extracts of *L. arabinosus* were prepared from cells grown in from 5 to 10 l. of complete Shiota[29] medium. The washed cells (from 20 to 27 g, wet weight) were suspended in about 55 ml of 0·1 M, pH 6·5 Sorensen's phosphate buffer and treated for 1 hr at 6 °C in the Raytheon sonic oscillator (model DF 101) with the addition of approximately 1·5 g of Carborundum powder no. 240. The sonicates were clarified by centrifugation at 35,000 **g** for 20 min in the cold. The sulfosalicylic acid method,[32]

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with crystalline bovine albumin as standard, showed the final protein concentration to be approximately 13 mg/ml.

Cell-free extracts of *E. coli* were prepared from cultures grown in Lascelles and Woods[9] medium. They were sonicated for 5 min without the inclusion of Carborundum powder, and then were treated as described above for *L. arabinosus*.

Neither the *L. arabinosus* nor the *E. coli* extracts remained active (with respect to the synthesis of folinic acid) for more than 2 weeks, even when they were kept in a freezer. Extracts of *E. coli*, strain B (either dialysed or undialysed), were of no value for this reaction system because of the high background of folinic acid activity contained in extracts prepared from this wild-type strain.

Following incubation at 37 °C in the Dubnoff metabolic shaking incubator under an atmosphere of 95 per cent nitrogen and 5 per cent carbon dioxide, the reaction systems were heated at 115 °C for 30 min and assayed for folinic acid with *Pediococcus cerevisiae* (ATCC 8081). (The washed-cell systems were treated with chicken pancreas conjugase, prepared by the method of Doctor and Couch,[33] before they were assayed for folinic acid.) The folinic acid synthesized has been reported in terms of the total, natural form. The washed-cell systems were incubated for 2½ hr in the Dubnoff incubator; the cell-free extract systems were incubated for from 3 to 5 hr.

Sources of the labeled compounds

The aminopterin-2-^{14}C was a gift of the Southern Research Institute, Birmingham, Alabama. Pyrimethamine-2-^{14}C was prepared in this laboratory. For further details on these tracers see Wood and Hitchings[4]. The folic acid was tritiated commercially, and purified by Dr. E. Bresnick of this laboratory.

RESULTS

Comparative effects of amethopterin and pyrimethamine on the growth and synthesis of folinic acid by S. faecalis, L. arabinosus *and* E. coli

It is well known that the folic acid-requiring bacteria, such as *S. faecalis* and *L. casei*, are extremely sensitive to the anti-folic acid drugs. Moreover, it is known that certain other bacteria which have no growth requirement for folic acid are relatively insensitive to the anti-folic acids (for examples see Introduction). *L. arabinosus* seems to occupy an intermediate position in this respect for it is capable of utilizing for growth either relatively high concentrations of folic acid or low concentrations of *p*-aminobenzoic or *p*-aminobenzoylglutamic acids[34] (although growth on folic acid may be a resultant of its prior degradation to *p*-aminobenzoylglutamic acid[35]).

A comparison has been made of the inhibitory effects of amethopterin and pyrimethamine on folinic acid-synthesis by whole cells and by cell-free extracts of a representative folic acid-requiring bacterium (*S. faecalis*), a non-folic acid-utilizing bacterium (*E. coli*) (see Introduction), and the intermediate type micro-organism, *L. arabinosus*. These data are presented in Table 1. The concentration of amethopterin required to inhibit by 50 per cent the production of folinic acid by cell-free extracts of these three species of bacteria ranged between 0·1 to 5 mμg/ml. However, the concentration of this drug needed to effect the same degree of inhibition of folinic acid-synthesis in the intact cells ranged between 0·05 mμg for *S. faecalis* to greater than 1000 mμg for *E. coli*. Similar differences between the intact and the disrupted cells were found with pyrimethamine (Table 1). From these results it may be tentatively

concluded that *E. coli* and *L. arabinosus* are less permeable to these drugs than *S. faecalis*.

The data in Table 2 show that the concentrations of amethopterin and pyrimethamine required to produce 50 per cent inhibition of the growth of the three representative bacteria directly paralleled those concentrations needed to inhibit the synthesis of folinic acid by the intact cells (Table 1). Also, it is shown that there was a

TABLE 1. INHIBITION OF FOLINIC ACID SYNTHESIS BY ANTIFOLIC ACID DRUGS: CELLS *VS.* EXTRACTS

Preparation	Drug	*S. faecalis*	*L. arabinosus*	*E. coli*
(a) Washed cells	Amethopterin	0·05*	30*	>1000*
(b) Extract	Amethopterin	0·1	0·5	5
(a) Washed cells	Pyrimethamine	10	7000	>10,000
(b) Extract	Pyrimethamine	10	45	33

* Concentration, in mμg per ml, of either amethopterin or pyrimethamine required for 50 per cent inhibition of folinic acid biosynthesis.

Reaction systems—(a) Washed-cell preparations. *S. faecalis*: N-depleted bacterial cells (2 mg dry weight), folic acid (100 mμg/ml), glucose (0·02 M), ascorbic acid (0·01 M), sodium formate (0·02 M), $MgCl_2 \cdot 6H_2O$ (0·01 M), and phosphate buffer (0·01 M, pH 6·5) in a final volume of 2·0 ml. *L. arabinosus*: Washed cells, glucose, ascorbic acid, $MgCl_2 \cdot 6H_2O$, sodium formate and phosphate buffer were employed at the same concentrations given for *S. faecalis*. *p*-Aminobenzoylglutamic acid (0·5 μg/ml) replaced folic acid. *E. coli*: Washed cells, glucose, ascorbic acid, $MgCl_2 \cdot 6H_2O$ and phosphate buffer were employed at the same concentrations given for *S. faecalis*. *p*-Aminobenzoic acid (0·5 μg/ml) and L-glutamic acid (0·006 M) were also included.

(b) Cell-free extract preparations. *S. faecalis*: Extract (5 mg protein), folic acid (2 μg/ml), TPNH (0·0003 M), ascorbic acid (0·003 M) and phosphate buffer (0·1 M, pH 6·5) in a final volume of 2·0 ml. *L. arabinosus*: Extract (1·2 mg protein), folic acid (2 μg/ml), $MgCl_2 \cdot 6H_2O$ (0·0025 M), ascorbic acid (0·003 M), sodium formate (0·0074 M), ATP (0·0005 M), TPNH (0·0007 M) and phosphate buffer (0·05 M, pH 6·5) in a final volume of 2·0 ml. *E. coli*: Extract (0·73 mg protein) plus folic acid, $MgCl_2 \cdot 6H_2O$, ascorbic acid, sodium formate, ATP and phosphate buffer at the concentrations given for *L. arabinosus*. Glucose (0·005 M) and TPN (0·0006 M) were also included.

corresponding increase in the amount of leucovorin needed to counteract the inhibition of growth by these concentrations of the anti-folic acid drugs. Thus, growth studies also serve to suggest that amethopterin, pyrimethamine and leucovorin penetrate with progressively less facility into *S. faecalis*, *L. arabinosus*, and *E. coli*, in this order.

A role for thiamin in the uptake of amethopterin by L. arabinosus

McGlohon *et al.*[1] have reported that folic acid and leucovorin are relatively ineffective in preventing the action of aminopterin on the growth of *L. arabinosus*, whereas the combination of thiamin plus pyridoxal is quite effective in this respect. We have repeated their growth experiments—however, using the richer medium of Shiota[29]—and have observed the same type of protection by these vitamins when amethopterin was substituted for aminopterin; as illustrated in Fig. 1, thiamin plus pyridoxal was the most effective combination. Thiamin alone was nearly as effective as the combination, while pyridoxal alone was ineffective. Folic acid or leucovorin was much less active than either the combination of thiamin plus pyridoxal or thiamin alone. Under the same experimental conditions none of these vitamins was active in counteracting the inhibition of growth of *L. arabinosus* produced by pyrimethamine (Fig. 1).

McGlohon *et al.*[1] interpreted the counteracting effect of thiamin and pyridoxal as evidence for the existence of an alternative pathway for the synthesis of polynucleotides "which has a limited dependence on folic acid compounds but is mediated in some way by B_1 and B_6". However, it seemed that there were other possible interpretations for the effects of thiamin. One possibility was that thiamin might interfere with the uptake of amethopterin and aminopterin. Accordingly, the effect of thiamin

Table 2. Inhibition of growth by the antifolic acid drugs: effect of leucovorin

Drug added	*S. faecalis*		*L. arabinosus*		*E. coli*	
	(a)	(b)	(a)	(b)	(a)	(b)
Amethopterin	0·1	<0·5	30	10,000	50,000	200,000
Pyrimethamine	3	<0·5	500	>10,000	8000	>200,000

(a) Concentration in mμg/ml of either amethopterin or pyrimethamine required to produce 50 per cent inhibition of growth.

(b) Concentration of leucovorin in mμg/ml required to counteract completely the inhibition produced by drug concentration given in column (a).

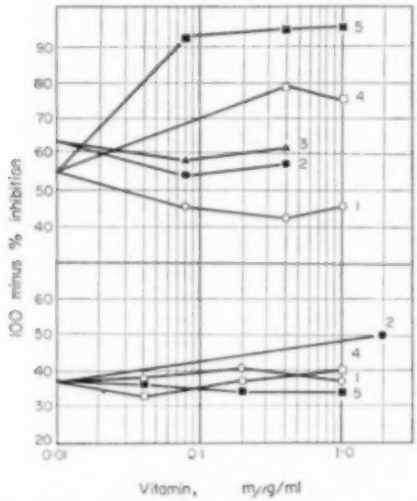

Fig. 1. Capacity of several vitamins to counteract the inhibition of the growth of *L. arabinosus* produced by amethopterin or pyrimethamine. *Upper* half of figure depicts the effects of the vitamins on the inhibition of growth produced by 30 mμg of amethopterin per ml, recorded at 41 hr of growth. *Lower* half depicts the effects of the vitamins on the inhibition produced by 500 mμg of pyrimethamine per ml, recorded at 23 hr of growth. *Curves*: no. 1 = pyridoxal; no. 2 = leucovorin; no. 3 = folic acid; no. 4 = thiamin; and no. 5 = thiamin plus pyridoxal. The medium contained 10 mμg of *p*-aminobenzoic acid per ml.

on the uptake of aminopterin-2-^{14}C by washed suspensions of *L. arabinosus* was investigated, with the results as given in Table 3. Thiamin inhibited markedly the assimilation of aminopterin by *L. arabinosus*. Since Guthrie *et al.*[36] have observed that thiamin and certain of its derivatives, but not folic acid or leucovorin,[21, 36] counteract the inhibitory action of amethopterin on the growth of *Bacillus subtilis*, the effect of

thiamin on the uptake of aminopterin-2-^{14}C by this species was also determined. Again, thiamin interfered with the uptake of aminopterin (Table 3). While our work was in progress Pine[43] reported the same interference by the pyrimidine moiety of thiamin with the uptake of aminopterin by *B. subtilis*.

TABLE 3. FACTORS AFFECTING THE UPTAKE OF FOLIC ACID AND ITS ANTAGONISTS

Uptake systems*	Radioactivity released from cells, counts/min per mg dry cells	
	L. arabinosus	*B. subtilis*
(a) Aminopterin-2-^{14}C	17·0	9·0
+ thiamin	10·0	3·8
+ glucose	202·0	10·8
+ glucose + thiamin	85·0	2·9
(b) Pyrimethamine-2-^{14}C	5·2	
+ thiamine	5·6	
+ glucose	2·3	
+ thiamin + glucose	2·3	
(c) Folic acid-^{3}H	6·1	
+ thiamin	6·5	
+ glucose	29·2	
+ glucose + thiamin	25·2	

* The uptake systems contained (where indicated) in a total volume of 3·0 ml:

(a) Aminopterin-2-^{14}C (3·0 μg; specific activity = 12,333 counts/min per μg) + washed bacterial cells (6·5 mg (*B. subtilis*) or 8·5 mg (*L. arabinosus*) dry weight) + glucose (1·5 per cent) + thiamin (25 μg) + Sørensen's phosphate buffer (0·05 M, pH 6·4).

(b) Pyrimethamine-2-^{14}C (30·0 μg; specific activity = 235 counts/min per μg) + washed cells (8·5 mg dry weight) + glucose (3 per cent) + thiamin (30·0 μg) + phosphate buffer (0·1 M, pH 7·4).

(c) Folic acid-^{3}H (15 μg; specific activity = 3500 counts/min per μg) + washed cells (8·5 mg dry weight) + glucose (3 per cent) + thiamin (30 μg) + phosphate buffer (0·1 M, pH 6·5).

The systems were incubated aerobically for 30 min at 37 °C. Then the cells were washed three times with buffer and resuspended in 1 ml of water. This was followed by heating at 120 °C for 10 min. The cells were removed by centrifugation and the entire supernatant fraction was dried on a planchet and counted at infinite thinness in a gas flow counter.

TABLE 4. INHIBITION OF FOLINIC ACID BIOSYNTHESIS BY THE ANTIFOLIC ACID IN *L. arabinosus*: EFFECT OF THIAMIN

Reaction system*	Folinic acid synthesized, mμg/mg dry cells	Percentage inhibition
Control	99·0	
+ thiamin	106·5	
+ amethopterin	46·5	53·0
+ amethopterin + thiamin	97·5	8·5
+ pyrimethamine	61·5	37·8
+ pyrimethamine + thiamin	66·0	38·0

* The basal reaction system was exactly as given in Table 1 for washed-cell preparations of *L. arabinosus*. Where indicated, amethopterin (20 mμg/ml), pyrimethamine (10 μg/ml) and thiamin (10 μg/ml) were added to the basal reaction system.

Just as thiamin was incapable of counteracting the inhibition of the growth of *L. arabinosus* produced by pyrimethamine (Fig. 1), so also was thiamin without effect on the uptake of pyrimethamine-2-^{14}C by this bacterium (Table 3). Interestingly, thiamin had no effect on the uptake of folic acid-^{3}H (Table 3).

To determine the influence of thiamin on the degree of inhibition of folinic acid synthesis produced by amethopterin and pyrimethamine, cells of *L. arabinosus* were harvested from media lacking thiamin. These cells were then used in the usual folinic acid synthesis system with *p*-aminobenzoylglutamic acid as the limiting substrate. The results of this experiment are presented in Table 4. The addition of thiamin to the reaction system lowered the inhibition by amethopterin from 53 per cent to 8·5 per cent*. There was no effect of thiamin on the inhibition of folinic acid synthesis produced by pyrimethamine.†

It may be concluded from the foregoing experiments that thiamin counteracts the inhibition of the growth of *L. arabinosus* produced by amethopterin by preventing its uptake by the cell. This in turn interferes with the subsequent effect of amethopterin on the intracellular synthesis of folinic acid. It can safely be assumed that amethopterin does not inhibit the growth of *L. arabinosus* by interfering with the uptake of thiamin, as this bacterium can grow readily in the absence of added thiamin.

Influence of pH on the inhibition of L. arabinosus *by pyrimethamine*

During the course of this study it was noted empirically that slight variations in the pH to which the medium had been adjusted prior to sterilization profoundly influenced the subsequent degree of growth inhibition produced by pyrimethamine or

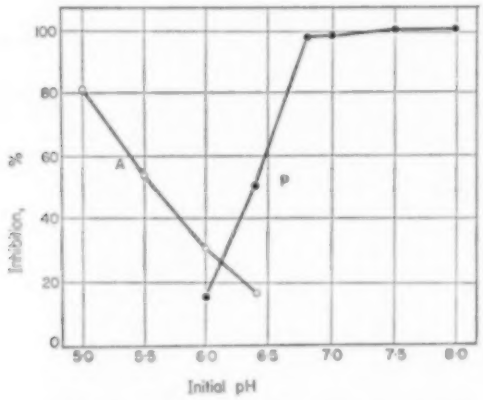

Fig. 2. Effect of the pH to which the growth medium had been adjusted prior to sterilization on the subsequent degree of growth inhibition of *L. arabinosus* produced by amethopterin or pyrimethamine. Curve A depicts the effects of 30 mμg of amethopterin per ml on growth. Curve P depicts the effects of 500 mμg of pyrimethamine per ml on growth. Turbidities were recorded at 18 hr of growth in a medium containing 10 mμg of *p*-aminobenzoic acid per ml.

amethopterin. This effect is illustrated in Fig. 2. A drop in the pH of the medium decreased the inhibition of growth caused by pyrimethamine; it increased the inhibition by amethopterin. It was considered possible that the pH of the medium might influence the cellular penetration of the drugs. This possibility was investigated in

* Pine and Guthrie[38] found that the pyrimidine moiety of thiamin was able to prevent the inhibitory effect of amethopterin on the incorporation of formate-^{14}C by *B. subtilis*.

† In washed-cell experiments with *S. faecalis* thiamin (1 μg/ml) was unable to counteract the inhibition of folinic acid-synthesis produced by either amethopterin (10 mμg/ml) or pyrimethamine (20 mμg/ml) (unpublished observation).

detail only in the case of pyrimethamine because the supply of aminopterin-2-^{14}C had become exhausted by this time.

If the effect of pH on the inhibition of growth by pyrimethamine were actually a reflection of its influence on the uptake of the drug, then the uptake of pyrimethamine by washed cells should be similarly related to the pH of the uptake reaction system. This was tested directly by incubating washed suspensions of *L. arabinosus* with pyrimethamine-2-^{14}C at several pH values. This experiment is depicted in Fig. 3.

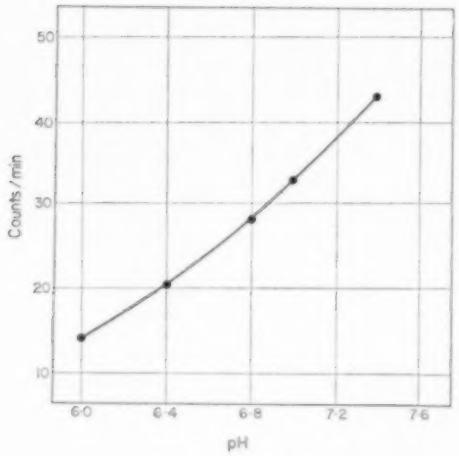

FIG. 3. Effect of pH on the uptake of pyrimethamine-2-^{14}C by *L. arabinosus* cells. The 3·0-ml uptake systems contained 8·5 mg dry weight of washed cells, 30 μg of labeled drug and 0·1 M Sorensen's phosphate buffers of the pH values indicated. The systems were incubated at 37 °C for 20 min and then the cells were washed once with the same buffer in which they had been incubated. The systems were treated then as described in Table 3.

There was a definite rise in the retention of the drug with increasing pH, demonstrating the desired relationship between inhibition and uptake.

The pH of the medium influences the growth-inhibitory action of pyrimethamine on *L. arabinosus*. From this it should follow that the pH should affect in a similar fashion the inhibition by pyrimethamine of the synthesis of folinic acid, as this is presumably the means by which pyrimethamine inhibits this bacterium (see Table 1). The effect of pH on the synthesis of folinic acid by whole cells and by cell-free extracts of *L. arabinosus* was determined, with the results as depicted in Fig. 4. Pyrimethamine produced more inhibition of folinic acid synthesis by whole cells as the pH was raised, which was the same influence pH had on the inhibition of growth by the drug. In cell-free extracts, however, pyrimethamine exerted progressively more inhibition of folinic acid synthesis as the pH dropped.

There was some question as to whether pyrimethamine-2-^{14}C was actually assimilated by *L. arabinosus* cells, for it was found that heat-killed cells retained the same amount of radioactivity as the viable cells. The following experiment was performed to determine whether the drug was actually assimilated by *L. arabinosus* or whether it exerted its action merely by binding with the surface of the cell.

Washed cells of *L. arabinosus* were permitted to take up *p*-aminobenzoylglutamic acid in the absence of pyrimethamine. Then the cells were washed and added to the usual folinic acid-synthesis system containing pyrimethamine, but lacking *p*-aminobenzoylglutamic acid. It was observed that 7 μg of pyrimethamine per ml produced a 58 per cent inhibition of the conversion of the intracellular *p*-aminobenzoylglutamic acid to folinic acid. This was the same concentration of drug required to inhibit by 50 per cent the conversion of extracellular *p*-aminobenzoylglutamic acid to folinic acid (see Table 1). Evidently, then, pyrimethamine does actually enter the cell and

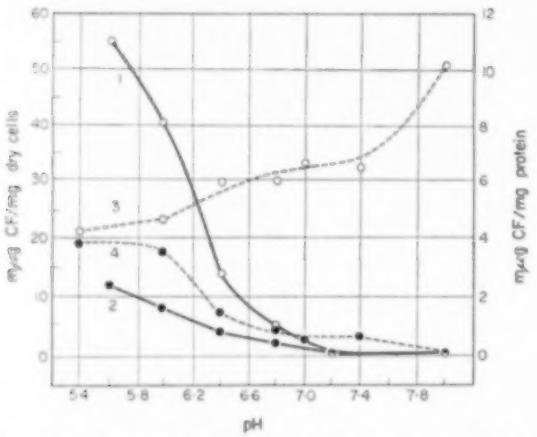

Fig. 4. Effect of pH upon the inhibition of folinic acid synthesis produced by pyrimethamine in *L. arabinosus*: a comparison of whole cells and cell-free extracts. *Extracts*: Curve no. 1 depicts the control set; curve no. 2 depicts the set containing 40 mμg of pyrimethamine per ml. With the exception of the inclusion of glucose (0·006 M) in this experiment, the same reaction system as that described in Table 1 for the *L. arabinosus* extract was employed here. *Whole cells*: Curve no. 3 depicts the control set; curve no. 4 depicts the set containing 7 μg of pyrimethamine per ml. The components of the reaction systems were identical with those described in Table 1 for the *L. arabinosus* washed-cell preparations. "CF" means folinic acid.

inhibit the conversion of endogenous *p*-aminobenzoylglutamic acid to folinic acid. Therefore, the equivalent uptake of pyrimethamine-2-^{14}C by living and by dead cells of *L. arabinosus* appears to be similar to the situation in which dead micro-organisms, e.g. yeast cells,[39] have been observed to absorb quantitative amounts of dyes such as methylene blue.

DISCUSSION

There is a tremendous range in the concentrations of amethopterin and pyrimethamine required to produce inhibition of the growth of the three species of bacteria examined in this study. However, the basic site of action of these drugs seems to be the same in all cases, viz. the conversion of folic acid to folinic acid. Assuming that the reaction studied in the extract experiments is the natural one carried out by the cell, then the profound differences in sensitivity to the drugs seem to lie in species differences in permeability to the drugs and not in differences in the sensitivity of their intracellular enzymes.

The uptake characteristics of *L. arabinosus* are unusual. It can assimilate amethopterin but not equivalent concentrations of folic or folinic acids. The reason for this

seems to be that the transport of amethopterin, but not that of the folic acids, is facilitated by a system which normally takes up thiamin. This transport system works best in the presence of an energy source (glucose) and would, by definition, be termed an active transport system.[40] Since thiamin interferes with the uptake of aminopterin, but not of pyrimethamine, only the transport of aminopterin (and presumably amethopterin) would seem to be mediated by this system. This transport system seems to have a high degree of specificity, since the uptake of folic acid, which differs from aminopterin only in the substitution of a hydroxyl group for an amino group, is not influenced by thiamin. Perhaps the presence of a 4-amino group on the pteridine ring is necessary for the molecule to fit the site on the cell surface where thiamin and aminopterin are absorbed. Pyrimethamine seems to enter the cell via a passive transport system, since glucose inhibits its uptake (Table 3) and cellular viability is unnecessary. Its uptake is influenced principally by its degree of ionization (see below).

According to general theory,[41] the state of ionization of an ionizable drug should influence its uptake and also its subsequent effect within the cell. The drug must be relatively undissociated in order to enter the cell. Within the cell, the ionized form should be the more active. The results of the experiments dealing with the effect of pH on the action of pyrimethamine on intact cells as compared with cell-free extracts fit this theory. Pyrimethamine is absorbed best and is most inhibitory toward the intact cells at pH values near its pK_a (7·2*); it is most effective against the extracts at the lower pH values, at which it is more dissociated.

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The anti-folic acid drugs seem to occupy a unique position among the antimetabolites. They are extremely potent drugs and their action on the conversion of folic acid to folinic acid is non-competitive,[31] making their reversal with folic acid extremely difficult. Furthermore, the reaction catalyzing the reduction of folic acid is sensitive to many drugs which are not very similar structurally to folic acid. However, there is yet another quality of the folic acid system which makes it of value from the standpoint of selective chemotherapy. The capacity to take up folic acid and its derivatives evidently is present only in certain cells. When this transport system is lacking, it usually requires a concentration of these metabolites which is at least 1000-fold higher than the physiological concentration before they can be forced into the cell. Since the anti-folic acid drugs such as amethopterin or aminopterin generally enter via the folic acid transport system,[4] only those cells which can assimilate the vitamin will be affected by the drugs.

Several different interrelationships between the cellular transport of a metabolite and its corresponding antimetabolite seem to exist in nature. One case is that in which both are assimilated with facility. This is illustrated by the uptake of folic acid, folinic acid and their analogs by *S. faecalis* and *L. casei*.[4] A second situation is one in which only the antimetabolite is assimilated, as with the folic acid analogs in *B. subtilis*.[21, 37, 42] Another conceivable situation is one in which only the metabolite is taken up. An example of this is unknown. Finally, there is the situation in which neither the metabolite nor the antimetabolite can be assimilated. This apparently applies to *E. coli*, which cannot take up folic or folinic acids[4, 12, 15] and is inhibited only by extremely high concentrations of the anti-folic acid drugs.[11, 23–27]

As more and more data are gathered concerning the complement of intracellular enzymes possessed by various species, it is becoming increasingly apparent that there

* Unpublished observation of Bases and Hitchings.

is a far greater degree of enzymatic similarity among species than there is dissimilarity. That is, the intracellular enzymic differences are quantitative rather than qualitative. On the other hand, it is possible to observe what might be called for practical purposes a qualitative difference in the permease constitution of various bacterial species. This means that practical chemotherapy may be able to exploit these differences.

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THE EFFECT OF TRIETHYLTIN ON OXIDATIVE PHOSPHORYLATION AND MITOCHONDRIAL ADENOSINE TRIPHOSPHATASE ACTIVATION

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Abstract—Triethyltin inhibits the respiration of rat brain slices following *in vitro* and *in vivo* administration. When added *in vitro* this alkyltin compound uncouples oxidative phosphorylation of rat brain and liver homogenates and mitochondria. Triethyltin also inhibits both the magnesium and the 2:4-dinitrophenol activated adenosine triphosphatases of isolated rat liver mitchondria.

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ALKYLTIN compounds are extremely toxic materials.[1] Their indiscriminate use in France for the treatment of furunculosis resulted in the unfortunate death of over one hundred persons.[2] During the course of our study on the toxicity of triethyltin acetate we observed that this compound had many interesting biochemical properties. We have confirmed the work of Aldridge and Cremer[3] and of Aldridge[4] that this compound inhibits brain slice respiration and is capable of uncoupling oxidative phosphorylation at concentrations comparable to those that are found in tissues after this agent is injected into rats. In contrast to the work of Aldridge[4] we have found that triethyltin (TET) is incapable of accelerating the rate of oxidation of acceptor-deficient mitochondrial systems and inhibits both the latent or magnesium-activated adenosine triphosphatase and the dinitrophenol-activated adenosine triphosphatase. The significance of these findings relative to the toxicity of this compound and its use as a biochemical research tool will be considered in the present communication.

EXPERIMENTAL MATERIALS AND METHODS

Female rats (Sprague–Dawley) weighing approximately 200 g were used in this study. All preparative procedures were carried out in a 5 °C cold room. The animals were sacrificed by decapitation and slices were prepared from liver and cerebral cortex by the method of Deutsch.[5] Two slices (total weight approximately 100 mg) were weighed and placed in Warburg vessels containing $1{\cdot}27 \times 10^{-1}$ M NaCl, $5{\cdot}1 \times 10^{-3}$ M KCl, $1{\cdot}27 \times 10^{-3}$ KH_2PO_4, 1×10^{-2} M Na_2HPO_4 pH 7·4, $1{\cdot}36 \times 10^{-3}$ M $CaCl_2$, $1{\cdot}27 \times 10^{-3}$ M $MgSO_4$ and $1{\cdot}1 \times 10^{-2}$ M glucose. The total volume was 2·0 ml and the center well contained 0·2 ml 2N KOH with filter paper. The gas phase was oxygen and the temperature 37 °C. After an equilibration of 10 min the oxygen consumption was determined for a 30-min period.

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Oxidative phosphorylation was performed on brain and liver homogenates and mitchondria by the method of Brody and Bain.[6] Twenty per cent homogenates of liver and brain were prepared by the method of Potter and Elvehjem.[7] Liver mitochondria were isolated according to the procedures described by Hogeboom *et al.*[8] so that the final concentration contained mitchondria from 500 mg wet weight liver per ml. Brain mitochondria were prepared as described by Brody and Bain[9] so that 1 ml of the final concentration contained the mitochondria from 1 g of brain. Each Warburg vessel contained $2{\cdot}5 \times 10^{-2}$ M potassium phosphate buffer pH 7·4, $1{\cdot}1 \times 10^{-2}$ M glycylglycine buffer pH 7·4, $1{\cdot}2 \times 10^{-2}$ M KCl, $1{\cdot}25 \times 10^{-3}$ M $MgCl_2$, $2{\cdot}8 \times 10^{-3}$ M dipotassium adenosine triphosphate (ATP), $1{\cdot}0 \times 10^{-5}$ M cytochrome *c*, 25 units yeast hexokinase (Pabst), $3{\cdot}3 \times 10^{-2}$ M glucose, 0·4 ml mitochondrial suspension or homogenate and $1{\cdot}5 \times 10^{-2}$ M NaF. Substrate concentrations are given in the individual tables. The total volume of the reaction mixture was 1·9 ml while the side arm contained 0·1 ml 75 per cent trichloroacetic acid and the center well 0·2 ml 2N KOH. The temperature was 30 °C and the gas phase was air. After temperature equilibration of the flasks for 9 min they were incubated for a period of 10 min.

The 2:4-dinitrophenol activated adenosine triophosphatase (DNP ATPase) was determined in individual test tubes containing 5×10^{-2} M Tris buffer at various pH values, $2{\cdot}5 \times 10^{-2}$ M KCl, $1{\cdot}47 \times 10^{-1}$ M sucrose, 1×10^{-3} M disodium ethylenediaminetetra-acetic acid (EDTA), 1×10^{-4} M 2:4-dinitrophenol (DNP) and mitochondria equivalent to 50 mg liver. For the magnesium-activated adenosine triphosphatase (Mg ATPase) the conditions were the same except that the DNP and EDTA were omitted and instead the tubes contained 5×10^{-3} M $MgCl_2$. After thermal equilibration, ATP (final concentration $2{\cdot}5 \times 10^{-3}$ M) was added to the tubes so that

Table 1. Effect of *in vitro* triethyltin on brain slice respiration

Additions	Number of expts.	Q_{O_2}*	% of control
Control	17	76·0 ± 9·2	100
TET 10^{-7} M	11	82·2 ± 10·0	108
TET 10^{-6} M	12	56·3 ± 7·9	72·7†
TET 10^{-5} M	8	29·2 ± 8·3	38·4†
TET 10^{-4} M	4	10·9 ± 2·6	14·4†

* Mean ± standard deviation = μM_{O_2}/g wet weight/hr.
† Significantly different from control at less than 1 per cent confidence level.

the final reaction volume was 1·8 ml. The reaction was stopped at zero and 15 min by the addition of 0·2 ml of 50 per cent trichloroacetic acid. All experiments were performed at 30 °C. Inorganic phosphate was determined by the method of Fiske and SubbaRow.[10]

RESULTS

1. *Tissue slice respiration*

Initial studies with TET were performed on brain slices since Aldridge and Cremer[3, 11] reported that the organo-tin compounds were potent inhibitors of brain but not liver slices. As seen in Table 1 TET significantly reduces the respiration of rat

brain slices at concentrations as low as 10^{-6} M. This inhibitory action was more pronounced as the concentration of TET increased.

Confirming the results of Cremer[11] it was found that liver slices were not profoundly inhibited by the *in vitro* actions of TET. In three determinations at 10^{-4} M TET the liver slice respiration was reduced by 23, 30 and 36 per cent and at 10^{-5} M TET by only 9, 10 and 7 per cent. When compared with a reduction of respiration in brain slices of 85 per cent at 10^{-4} M and 60 per cent at 10^{-5} M TET (Table 1) it is evident that the brain is somewhat more sensitive to the inhibitory actions of TET.

Following injection of triethyltin acetate (10 mg/kg) into rats a decrease in brain but not in liver slice respiration of treated animals was observed (Table 2). In all but

Table 2. EFFECT OF *in vivo* TRIETHYLTIN ON BRAIN AND LIVER SLICE RESPIRATION

Hours after injection	Route	Mean % of control O_2 uptake	
		Brain	Liver
2	i.p.	*63 (40– 78)	—
2	i.p.	87 (82– 91)	103 (98–114)
2	i.p.	103 (93–117)	101 (93–110)
2	i.p.	74 (69– 80)	119 (102–142)
2	i.p.	85 (74–100)	115 (100–138)
4	i.p.	83 (76– 89)	106 (89–117)
6	i.p.	77 (59– 86)	94 (70–124)
2	s.q.	96 (95– 98)	—
4	s.q.	99 (93–107)	101 (86–112)
6	s.q.	72 (66– 79)	108 (99–116)

* Figures represent the mean and range of the percentage of the Q_{O_2} of slices removed from rats receiving 10 mg/kg triethyltin acetate when compared with the Q_{O_2} of slices removed from control animals. Each mean represents the percentage calculated from two flasks containing control slices and four flasks containing treated slices. i.p. = intraperitoneal and s.q. = subcutaneous route of injection.

one experiment the brain slice respiration was decreased while that of the liver slices was often slightly increased 2 hr after intraperitoneal injection. By the subcutaneous route a longer time after injection (6 hr) was required before inhibition could be detected. This was probably due to the time required for the concentration of TET in the brain to reach adequate levels. That brain slice respiration was more selectively sensitive to TET was also indicated by TET tissue distribution studies. Analyses of tissues for TET 2 hr after injection revealed from 5 to 10 times as much TET in the liver as in the brain (Moore and Brody, unpublished), yet liver slice respiration was not depressed. This finding has also been reported by other investigators.[11]

2. *Homogenate and mitochondrial oxidative phosphorylation*

Although brain slices appeared to be much more sensitive than liver slices to the inhibitory actions of TET, mitochondria and homogenates from both brain and liver were equally affected by TET *in vitro*. As seen in Table 3 the respiration of both brain and liver homogenates was markedly inhibited by 10^{-5} M TET. Glutamate oxidation appeared to be more sensitive to the actions of this compound than did the succinoxidase system since at 10^{-4} M TET, glutamate oxidation was reduced by 80–90 per cent

whereas succinate oxidation was reduced by only 30–50 per cent. Although this difference occurred between these two systems with respect to respiration, the phosphorylating ability with both substrates was equally depressed. In the isolated mitochondrial preparations the inhibition of respiration and uncoupling of oxidative phosphorylation was similar to that observed in the homogenate preparations.

Table 3. Effect of *in vitro* triethyltin on oxidative phosphorylation

Preparation	Substrate		Triethyltin concentration				
			None	10^{-6} M	10^{-5} M	5×10^{-5} M	10^{-4} M
Brain homogenate	G	Q_{O_2}	3·76	3·38	2·78	1·15	0·77
		P/O	1·92	1·43	1·26	0·22	0
	S	Q_{O_2}	4·63	4·35	4·02	2·93	3·03
		P/O	1·10	1·16	0·93	0	0
Liver homogenate	G	Q_{O_2}	8·83	8·61	6·32	2·53	0·81
		P/O	1·99	1·95	1·63	0·92	0
	S	Q_{O_2}	10·70	10·80	8·90	5·40	5·40
		P/O	1·13	1·21	0·95	0·30	0
Brain mitochondria	G	Q_{O_2}	3·12	3·18	1·46	0·77	—
		P/O	2·54	2·63	1·12	0	—
	S	Q_{O_2}	4·69	4·49	3·50	3·13	—
		P/O	1·66	1·16	0·57	0·13	—
Liver mitochondria	G	Q_{O_2}	5·43	—	2·46	0·10	0·11
		P/O	2·79	—	1·62	0	0
	S	Q_{O_2}	8·07	—	8·11	5·87	—
		P/O	1·74	—	1·03	0·20	—

G = glutamate, S = succinate; both at substrate concentrations of 0·01 M.
Q_{O_2} = microatoms of oxygen uptake.
P/O = ratio of micromoles phosphate uptake to microatoms of oxygen uptake.

Because of the apparent greater sensitivity of the diphosphopyridine (DPN)-linked dehydrogenase system to the *in vitro* actions of TET, the effects of DPN addition to there action medium was studied. Table 4 illustrates the ability of DPN partially to restore the oxidative activity of isolated mitochondria. TET markedly depressed oxygen uptake which was restored to approximately half of the control level by the addition of DPN. The apparent loss of this cofactor from the mitochondria, perhaps as a consequence of mitochondrial swelling, may account for the greater inhibition of glutamate oxidation than of succinoxidase activity seen with TET.

In order to determine if the uncoupling action of TET as seen in the *in vitro* systems could also be demonstrated in the whole animal, homogenates and mitochondria were prepared from brains and livers of rats injected with this compound. Despite the potent uncoupling ability of TET added *in vitro* we were unable to detect any uncoupling effects in mitochondria and homogenates prepared from brain and liver of TET treated rats. The reason for the failure to see uncoupling in these mitochondria is apparently the result of a decrease in TET concentration during the washing of the mitochondria. In Table 5, preparation of the mitochondria in isotonic sucrose containing 10^{-4} M TET resulted in the same typical effects of this compound when the

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same concentration was added directly to Warburg vessels (Table 3). However, washing the mitochondria once with 0·25 M sucrose prevented to a great extent the inhibitory actions of TET.

3. *Adenosine triphosphatases*

The actions of TET on liver mitchondrial ATPases are shown in Figs. 1 and 2. Freshly prepared normal mitochondria show very little Mg ATPase activity but this

TABLE 4. DIPHOSPHOPYRIDINE NUCLEOTIDE (DPN) REQUIREMENT FOR TRIETHYLTIN TREATED MITOCHONDRIA

Triethyltin concentration	Glutamate 0·01 M		Glutamate 0·01 M plus DPN 0·001 M	
	Q_{O_2}	P/O	Q_{O_2}	P/O
None	6·90	2·67	7·23	2·66
5×10^{-5} M	0·33	0	3·64	1·09

Conditions are same as described in methods except that triethyltin and DPN are added as indicated. Designations for Q_{O_2} and P/O are the same as in Table 3.

TABLE 5. EFFECTS OF WASHING MITOCHONDRIA ON THE INHIBITORY ACTION OF TRIETHYLTIN

Preparation of mitochondria		Glutamate 0·01 M	Glutamate 0·01 M plus DPN 0·001 M	Succinate 0·01 M
(1) Liver homogenized and washed in 0·25 M sucrose plus 10^{-4} M TET but final suspension made in 0·25 M sucrose.	Q_{O_2}	0	2·74	3·76
	P/O	0	0·88	0·64
(2) Liver homogenized in 0·25 M sucrose plus 10^{-4} M TET but washed and suspended in 0·25 M sucrose	Q_{O_2}	2·64	3·76	4·50
	P/O	3·01	2·34	1·95
(3) (Control) Liver homogenized, washed and suspended in 0·25 M sucrose.	Q_{O_2}	3·87	4·32	5·90
	P/O	2·48	2·78	2·04

Designations for Q_{O_2} and P/O are the same as in Table 3.

enzyme becomes markedly activated following damage to the mitochondria. The DNP ATPase on the other hand shows the reverse properties; it is markedly active in normal mitochondria but absent in damaged mitochondria. TET is capable of inhibiting both the DNP ATPases and the Mg ATPases. The actions on the Mg ATPase were studied on liver mitochondria from rats treated for 20 hr with carbon tetrachloride (2·5 ml/kg orally) or on mitochondria which had been treated with digitoxin (0·5 per cent). The spectrum of ATPase activity at different pH values was essentially the same as that reported by Myers and Slater[12] and Purvis[13]. The Mg ATPase spectrum was essentially the same regardless of the manner by which the mitochondria were damaged. The enzymic hydrolysis of ATP at pH 6·5 appeared less

sensitive to the actions of TET than that at the higher pH values. Whether this selectivity was the result of a differential action of TET on the specific ATPase as proposed by Myers and Slater[12]; or whether it resulted from differences in the ionization and subsequent penetration of TET into active mitochondrial enzyme sites is not known at the present time.

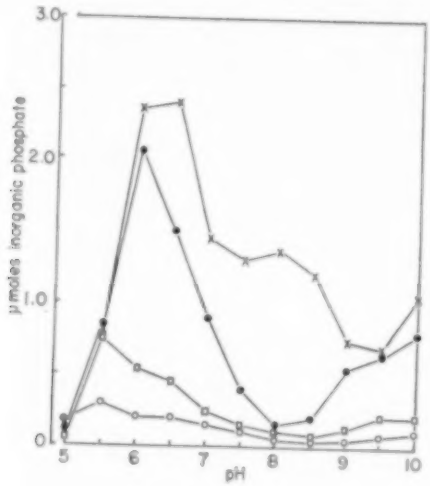

Fig. 1. Effect of triethyltin on the 2:4-dinitrophenol activated adenosinetriphosphatases. Concentrations of triethyltin are: ×—× none, ●—● 10^{-6} M, □—□ 10^{-5} M, ○—○ 10^{-4} M.

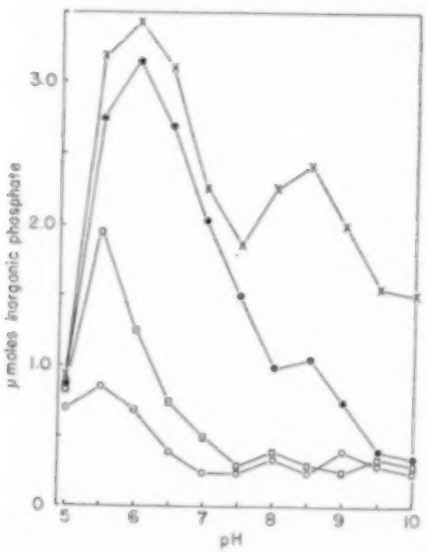

Fig. 2. Effect of triethyltin on the magnesium activated adenosinetriphosphatases. Concentrations of triethyltin are: ×—× none, ●—● 10^{-6} M, □—□ 10^{-5} M, ○—○ 10^{-4} M.

Contrary to the findings of Aldridge[4] no activation of the latent ATPase by TET could be observed either *in vivo* or *in vitro*. Nor was any acceleration of the respiration of mitochondria in an acceptor deficient system noted following *in vitro* treatment with this compound. TET inhibited the oxygen consumption in the acceptor-deficient, complete phosphorylating and DNP stimulated systems (Table 6).

TABLE 6. EFFECT OF *in vitro* TRIETHYLTIN ON OXIDATION BY LIVER MITOCHONDRIA IN ACCEPTOR-DEFICIENT SYSTEM

		Control	TET 10^{-6} M	TET 10^{-5} M
Experiment no. 1	No addition	1·80*	1·76	1·25
	DNP 10^{-5} M	2·70	2·87	2·15
Experiment no. 2	No addition	1·86	1·37	1·13
	hexokinase and glucose	5·86	5·26	3·39

* $Q_{O_2} = \mu AO_2/10$ min.

Conditions are as stated in methods except that hexokinase and glucose are omitted except where indicated. Glutamate 0·01 M is substrate.

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DISCUSSION

Studies with TET have been performed with a view to correlating the *in vitro* biochemical effects caused by this compound with the toxic actions that it produces in the intact animal. It was found that TET was a potent uncoupler of oxidative phosphorylation in rat liver and brain homogenates and mitochondria but that some of its properties differ from those of other uncouplers, i.e. DNP and thyroxine. The ability of TET to inhibit the respiration of both a complete phosphorylating system and an acceptor-deficient system and its ability to inhibit mitochondrial ATPases differentiate the actions of this compound from that of DNP. Like thyroxine, TET can produce mitochondrial swelling (see subsequent paper) at a concentration lower than that at which it is capable of uncoupling oxidative phosphorylation but unlike classical uncouplers TET produces a fall in the body temperature of rats.[14]

Aldridge[4] stated that although the activation of an ATPase was not the primary mode of action of TET, the ability of this compound to stimulate oxidation in an acceptor-deficient system paralleled its ability to stimulate the ATPases. We could find no such acceleration of respiration in an acceptor-deficient system by TET. The inhibitory action of TET on the mitochondrial ATPases and the ability of this compound to uncouple oxidative phosphorylation make it similar to the biochemical actions of sodium azide and *p*-hydroxymercuribenzoate.[15, 16] TET, however, appears to be at least ten times more potent than either of these latter two compounds.

Recently, there has been interest in the possibility that the DNP ATPase represents the phosphorylation associated with the diaphorase region of the electron transport system and that the Mg ATPase constitutes a part of the enzymes sequence involved in the DNP ATPase system.[16] It would appear that TET inhibits some step common to both pathways. TET must also uncouple at the other sites along the electron transport chain since the phosphorylations associated with succinate oxidation are markedly reduced. Because a portion of the TET molecule in solution exists as a

cation,[3] it is possible that it could accept electrons along the electron transport chain and thus divert their passage along the chain. The ability to participate in the electron transport chain could also account for the uncoupling ability of TET, since by accepting electrons and thereby setting up an alternate route for electron flow it is possible that such a pathway could exclude a step in the chain normally associated with energy-rich phosphate transfer. Aldridge and Cremer, however, could find no effect of TET on enzyme systems of the electron transport chain.[3]

By measuring ATPase activity of liver mitochondria at various hydrogen ion concentrations Myers and Slater[12] could identify three separate DNP ATPases which they proposed as representative of the enzymes which transferred the high-energy phosphate bonds from the various sites along the electron transport chain. However, more recent work[17] has revealed that there are no multiple pH optima for respiratory, phosphorylative and transfer reactions in liver mitochondria but instead only one single broad pH optima exists so that the identification of the various sites of phosphate transfer by this approach is not possible. The decreasing DNP ATPase activity with increasing pH suggests that the activation by DNP (pK_a 4·0) is associated with the undissociated phenol which decreases in concentration with increasing pH. However, Myers and Slater[12] feel that the active molecular species of DNP is the ionized form so that changes in pH affect only the enzyme sites rather than the inhibitor. This view does not consider the effect of pH upon the penetration of DNP into the mitochondrial sites. With most membranes the undissociated ion passes through with greater ease. Thus the greater activity of the DNP ATPase at the lower pH values could merely be the result of a greater concentration of DNP available at the active sites within the mitochondria since a greater quantity of the compound exists as the unionized form at these lower pH values. Likewise, the differential inhibition of the DNP ATPases and the Mg ATPase by TET (pK_a 6·5) at the various hydrogen ion concentrations may not be the selective inhibition of the ATPases with the higher pH optimum but merely an index of the penetration of TET into the active sites of the mitchondria since the concentration of the unionized species of TET will increase as the pH is increased. It is interesting to note that van der Kerk and Luijten[15] found the fungicidal activity of TET was more pronounced at higher pH values.

The correlation of biochemical properties of drugs which are observed *in vitro* with those actions of the drug which are seen in the intact animal is a difficult problem. Since the main action of TET appears to be on the central nervous system[2, 14] attention should be focused upon the biochemical changes that occur in nervous tissue. It was shown that TET inhibits the respiration of brain slices following *in vitro* and *in vivo* administration of the compound whereas it produces only slight inhibition of liver slice respiration *in vitro* and none *in vivo*. The exact mechanism of the decreased oxygen uptake by brain slices is not completely understood. It may be due to a loss of cofactors (e.g. DPN) or to an actual interference with the enzymes concerned with electron transport.

Concomitant with the decreased oxygen uptake by brain slices, the coupling of oxidation with high energy phosphate transfer in brain mitochondria and homogenates is disrupted by TET added *in vitro*. The combination of these two actions, i.e. decreased respiration and uncoupled oxidative phosphorylation, could result in a decrease in the energy reserves of the brain which would contribute to the toxicity of this agent. This hypothesis, however, is not strengthened by the findings of Stoner and

Threlfall[19] that there is no decrease in high energy phosphate containing compounds in animal tissues following TET administration.

Although a disruption of energy metabolism in the brain could account for many of the toxic actions of TET further study must be undertaken before these two effects of TET can be unequivocally associated.

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THE EFFECT OF TRIETHYLTIN ON MITOCHONDRIAL SWELLING*

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Abstract—Triethyltin induces swelling of isolated rat liver mitochondria suspended in sucrose or KCl media. This effect is not seen with brain mitochondria. Triethyltin swelling is only minimally reduced by EDTA or amytal but is prevented by 2:4-dinitrophenol, cyanide or salicylate. The addition of adenosine tri- or diphosphate causes reversal of the swelling; this effect is potentiated by Mn^{2+} but not by Mg^{2+}. The reversal of thyroxine-induced swelling by ATP is blocked by triethyltin, perhaps via an inhibition of the hydrolysis of adenosine triphosphate. The relationship between *in vitro* effects of triethyltin on mitochondria and the toxic actions of the drug in the whole animal are discussed.

IN THE past few years many investigators have studied the phenomenon of mitochondrial swelling.[1–8] Much of the interest has centered around the role of phosphate bond energy and oxidative phosphorylation in the maintenance of mitochondrial integrity. Substrates, cofactors, ions and more recently agents which uncouple oxidative phosphorylation have been examined for their effects upon mitochondrial swelling. Generally, there has been no correlation between uncoupling action and mitochondrial permeability; some uncoupling agents enhance while others protect against mitochondrial swelling.[2, 3]

The present work will demonstrate the effects of an organo-tin compound on mitochondrial permeability. This compound, triethyltin (TET) is an extremely toxic agent,[9, 10] a potent uncoupler of oxidative phosphorylation[11] and an inhibitor of mitochondrial adenosine triphosphatases (ATPases).[12] These studies were initiated with the view that such experiments might yield information pertinent to the elucidation of the phenomenon of mitochondrial swelling and further shed some light upon the mechanism of toxicity of the organo-tin compounds.

METHODS

Mitochondria were isolated from the livers of unstarved Holtzman female rats weighing 200–250 g by the method of Hogeboom *et al.*[13] Brain mitochondria were prepared as described by Brody and Bain.[14] The homogenization fluid was 0·25 M sucrose in those experiments in which swelling was determined in KCl-tris (hydroxymethyl) aminomethane (Tris) medium. When swelling was measured in sucrose-Tris medium, 0·3 M sucrose and 0·02 M Tris buffer pH 7·4 was used for the preparation and resuspension of the mitochondria. The mitochondria were prepared so that 1·0 ml of the final suspension contained the mitochondria from 0·5 g of liver. This stock suspension was kept at 0–2 °C and used within 2–3 hr after preparation. Swelling experi-

* Preliminary studies were reported in *Fed. Proc.* **19**, 139 (1960).

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ments were performed in KCl-Tris (0·125 M KCl–0·02 M Tris, pH 7·4) or in the sucrose–Tris medium described above. Other additions are designated in the individual figures. The total volume in each cuvette was 3·0 ml and additions of the mitochondrial stock solution (0·08–0·1 ml) were made prior to mixing and reading. With this mitochondrial suspension an initial optical density reading of from 0·500 to 0·600 was obtained at 520 mμ in the Beckman model DU spectrophotometer. After the initial reading the optical density values were recorded at 5 or 10 min intervals for 30 min. Incubation was at room temperature (22–24 °C). Additions to the cuvettes during the experiments were made in small volumes to minimize optical density changes.

RESULTS

1. *Effect of the suspending medium on TET-induced mitochondrial swelling*

Figs. 1 and 2 illustrate the ability of TET to induce swelling of isolated liver mitochondria in both KCl-Tris and sucrose-Tris buffer. Mitochondrial swelling, as evidenced by a decrease in the optical density values of a mitochondrial suspension, could

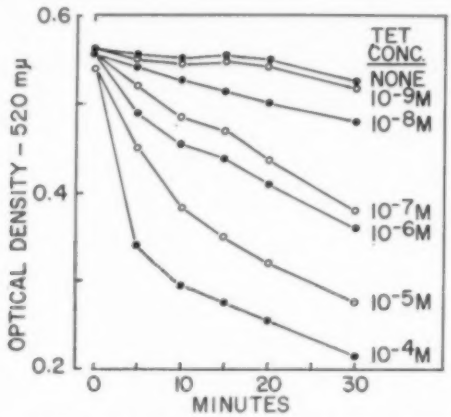

FIG. 1. Effect of TET on mitochondria suspended in 0·125 M KCl and 0·02 M Tris buffer, pH 7·4.

always be obtained with concentrations of TET as low as 10^{-7} M in KCl-Tris media and 10^{-6} M in sucrose-Tris. As the concentration of TET was increased, the effects upon swelling became more pronounced.

The osmolarity of the sucrose solution influenced the induction of swelling by TET. A sucrose concentration of 0·6 M reduced both the spontaneous and TET-induced swelling; but even in this medium some swelling was observed when TET was present (Fig. 3). In a solution of 0·15 M sucrose, the spontaneous swelling became very pronounced so that the effects of TET appeared to be less prominent.

Since the mitochondria appeared to be approximately ten times more sensitive to the swelling effect of TET in KCl-Tris buffer, most experiments were performed in this medium, although qualitatively similar results could be obtained when the experiments were repeated in sucrose-Tris buffer. All ATP-reversal studies were carried out in the KCl-Tris medium.

The optimum pH value for swelling with TET was between 7·5 and 8·0, which is approximately the same as that observed for spontaneous or thyroxine-induced swelling, but higher than that seen with reduced glutathione.[15]

2. *Ethylenediaminetetra-acetic acid (EDTA) on TET swelling*

Since it has been observed that many divalent heavy metal ions can cause mitochondrial swelling[3, 5] it was deemed advisable to determine whether TET-induced swelling could be the result of impurities of inorganic tin rather than an effect of the organic compound *per se*. As illustrated in Fig. 4 the addition of EDTA (final concentration, 10^{-4} M) at the beginning of the experiment or after 10 min only slightly reduced both TET-induced swelling and spontaneous swelling of mitochondria. Higher concentrations of EDTA (10^{-3} to 10^{-2} M) failed to block the TET-induced swelling to any significant extent.

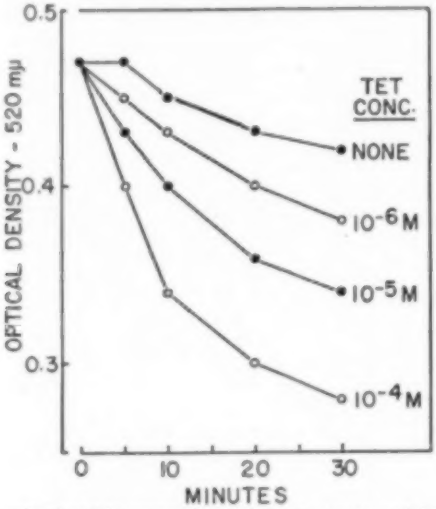

Fig. 2. Effect of TET on mitochondria suspended in 0·3 M sucrose and 0·02 M Tris buffer, pH 7·4.

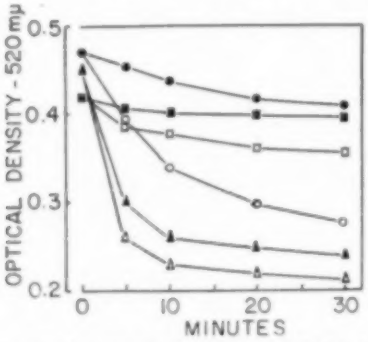

Fig. 3. Effect of sucrose concentration on spontaneous and TET-induced mitochondrial swelling. ●—control; and ○—10^{-4} M TET; in 0·3 M sucrose. ▲—control; and △—10^{-4} M TET; in 0·15 M sucrose. ■—control; and □—10^{-4} M TET; in 0·6 M sucrose.

3. *Blockade of adenosine triphosphate (ATP) reversal of thyroxine swelling*

Lehninger and co-workers have shown that thyroxine is capable of inducing a pronounced mitochondrial swelling in a KCl-Tris medium which can be reversed by the addition of ATP.[15, 16] It was also reported that such uncoupling agents as azide,

p-hydroxymercuribenzoate (formerly known as *p*-chloromercuribenzoate (PCMB)) and gramicidin inhibit the ATP reversal of swelling induced by thyroxine. As shown in Fig. 5 TET is also capable of blocking this reversal. This effect by TET was always prominent at a concentration of 10^{-6} M TET but only slight inhibition was evident at TET concentrations as low as 10^{-7} M. It was also observed that when TET was added during the process of thyroxine-induced swelling, there was only a slight additional swelling above that usually seen with thyroxine alone. Thus it would appear that the blockade of ATP reversal was a true inhibition and not merely a reflection of a greater swelling produced by the TET which might have masked the ATP reversal.

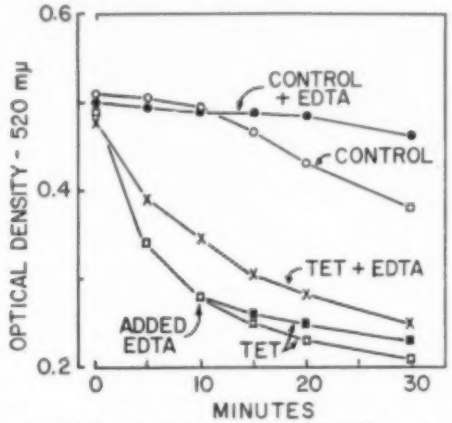

FIG. 4. Effect of EDTA on TET-induced mitochondrial swelling. The test system contained 0·125 M KCl and 0·02 M Tris buffer, pH 7·4 and 10^{-4} M EDTA and 10^{-4} M TET added as indicated.

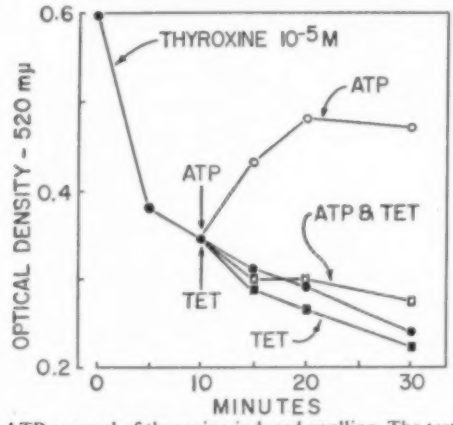

FIG. 5. Effect of TET on ATP reversal of thyroxine-induced swelling. The test system contained 0·125 M KCl and 0·02 M Tris buffer, pH 7·4; 1×10^{-5} M thyroxine and 3×10^{-3} M ATP and 1×10^{-6} M TET (final concentrations) added at 10 min as indicated by arrows.

4. *Reversal of TET swelling by ATP and ADP*

As demonstrated in Fig. 6 ATP has the ability to reverse mitochondrial swelling produced by low concentrations of TET (10^{-7} M). The addition of ATP to mitochondrial suspensions containing higher TET concentrations (10^{-6}–10^{-4} M) was not

effective in causing reversal. In Fig. 7 it may be observed that adenosine diphosphate (ADP) was less effective than ATP. The addition of adenosine monophosphate (AMP) could not reverse the TET-induced mitochondrial swelling. These data may be contrasted with the actions of the nucleotides on thyroxine-induced mitochondrial swelling, where ATP appears to be specific in its ability to reverse swelling. In our experiments the onset was immediate upon ATP addition, while the reversal seen with ADP was often slower in developing.

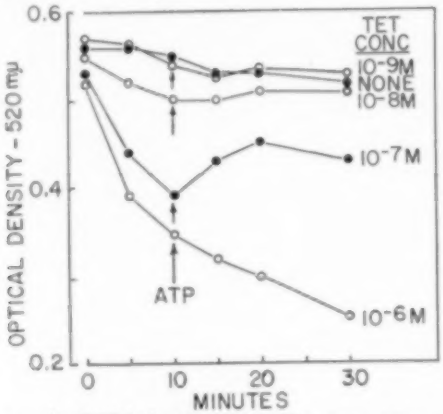

Fig. 6. Effect of ATP in reversing TET-induced swelling. The test system contained 0·125 M KCl and 0·02 M Tris buffer, pH 7·4. ATP (3×10^{-3} M, final concentration) was added as indicated by arrows.

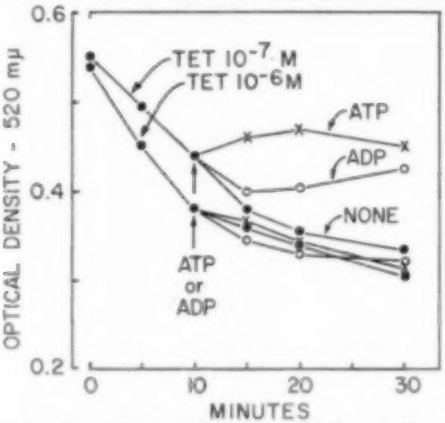

Fig. 7. Reversal of TET-induced swelling by ADP and ATP. Test medium contained 0·125 M KCl and 0·02 M Tris buffer, pH 7·4 with concentrations of TET as shown. ATP and ADP (final concentrations, 3×10^{-3} M) added as indicated by arrows. TET concentrations are final.

5. *Effect of manganese on TET-induced swelling*

It has been reported that mitochondrial swelling caused by many agents may be reversed by ATP if Mg^{2+} or Mn^{2+} are present in the medium. The reversal of thyroxine swelling by ATP requires neither Mg^{2+} nor Mn^{2+} ions.[17] If Mn^{2+} is added to the medium, ATP is now able to reverse the TET-induced swelling when the organo-tin compound is present in concentrations as high as 10^{-4} M (Fig. 8). Thus, the addition of Mn^{2+} markedly potentiates the reversing action of ATP. A similar potentation was

also observed with the addition of Mn^{2+} ions in studies with ADP. At a concentration of 3×10^{-3} M, $MgCl_2$ had no such effect on the reversal of TET swelling even though this ion has been reported to aid in reversing the swelling induced by other agents[17].

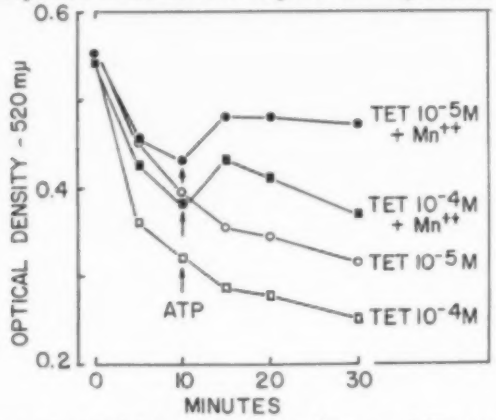

FIG. 8. Effect of manganese on TET-induced swelling and its reversal. The test medium was 0·125 M KCl and 0·02 M Tris buffer, pH 7·4, contained $MnCl_2$ (final concentration, 3×10^{-3} M) and ATP added at 10 min (final concentration 3×10^{-3} M). All TET concentrations are final.

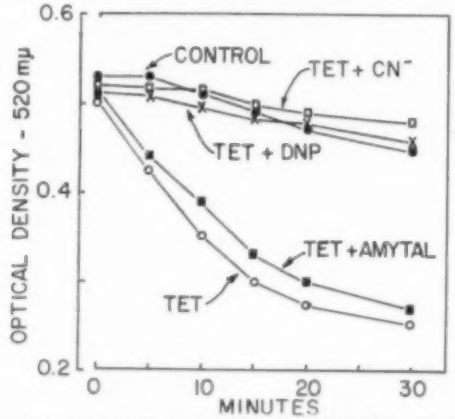

FIG. 9. The effect of respiratory inhibitors on TET-induced swelling. The test medium contained 0·125 M KCl and 0·02 M Tris buffer, pH 7·4 with 2×10^{-3} M NaCN, 2×10^{-3} M amytal and $3{\cdot}3 \times 10^{-5}$ M 2:4-dinitrophenol; 1×10^{-4} M TET added as indicated. All concentrations are final.

6. *Effect of electron transport chain blockade*

In an attempt to determine the role played by the various constituents of the transport chain several studies concerned with the effects of electron transport chain inhibitors on mitochondrial swelling have been reported.[18–20] As seen in Fig. 9, blockade of the electron transport chain by NaCN resulted in a protective effect upon TET swelling whereas no protection was observed with sodium amytal. The addition of 2:4-dinitrophenol (DNP) effectively prevented swelling induced by 10^{-4} M TET in sucrose-Tris and KCl-Tris media. Although not shown in the figure, 3×10^{-3} M sodium salicylate also prevented TET-induced swelling. Agents capable of blocking electron flow through the hydrogen transport system have been reported to block

mitochondrial swelling produced by such substrates as succinate, glutamate and β-hydroxybutyrate. TET does not block swelling induced by glutamate but rather enhances the swelling response, so that the effects of substrate and TET on swelling appear to be additive (Fig. 10). Similar effects were observed when swelling was induced by succinate.

7. *Studies on brain mitochondria*

The marked effect of TET on the nervous system and its ability to produce edema in this tissue[21, 22] prompted us to study the effects of TET on isolated brain mitochondria. It has been difficult to induce swelling *in vitro* by any means in brain mitochondria,[23] although a recent report has indicated that under special conditions slight swelling can apparently be produced in brain mitochondria.[24] We have been unable to observe any swelling in isolated rat brain mitochondria when TET was added *in vitro*.

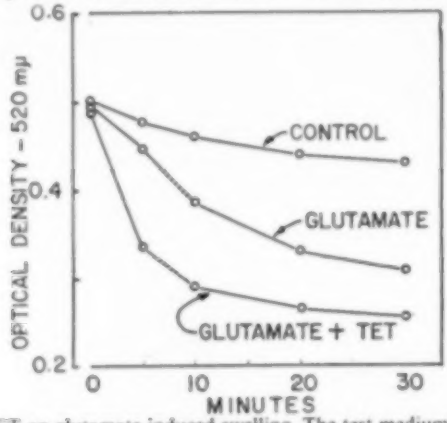

Fig. 10. The effect of TET on glutamate-induced swelling. The test medium contained 0·125 M KCl and 0·02 M Tris buffer, pH 7·4 and TET (1×10^{-4} M) and glutamate (3×10^{-3} M) as indicated. All concentrations are final.

DISCUSSION

TET, at low concentrations, induces swelling of isolated rat liver mitochondria suspended in KCl-Tris or sucrose-Tris buffers. Sucrose has been reported to be an unfavorable medium for studying mitochondrial swelling since sucrose solutions of varying osmolarity modify this phenomenon.[15] In the present study, however, qualitatively similar effects were observed in both KCl and sucrose media; the major difference was that the TET induced swelling was more pronounced and could be reversed by ATP in the KCl-Tris buffer.

Mitochondrial swelling has been produced by a number of metal ions such as Hg^{2+}, Ag^{+} and Zn^{2+}.[3, 17] The addition of EDTA reduces the swelling produced by these metals and by many other agents indicating that some other property of EDTA in addition to its chelating ability is responsible for this protection.[17] The mitochondrial swelling induced by TET, unlike the swelling produced by many other agents,[3] is not blocked by concentrations of EDTA as high as 10^{-2} M. It would appear therefore that the mitochondrial swelling observed following the addition of TET is the result of the action of the organo-tin compound *per se* and not to any impurity of inorganic tin ions since EDTA would be expected to protect the mitochondria from such metallic impurities.

There has been a great deal of work concerned with the effects of thyroxine on mitochondrial swelling. Dickens and Salmony[4] and more recently Lehninger *et al.*[15] have shown that thyroxine-induced swelling can be reversed by the addition of ATP to the medium. Water which enters the mitochondria during swelling is apparently extruded following the addition of ATP. To explain this phenomenon Lehninger[16] postulated an active contractile mechanism in the mitochondrial membrane that is associated with the hydrolysis of ATP. Since ATP reversal of swelling is blocked by azide, arsenate and PCMB he suggested that the contraction could be related in some way with the mechanism of oxidative phosphorylation. However, since ATP reversal of thyroxine swelling is not inhibited by cyanide, antimycin A nor DNP, neither respiration nor coupled phosphorylation is apparently required for the contractile mechanism. It was demonstrated that 10^{-6} M TET blocks the ATP reversal of thyroxine swelling. Since TET, like azide and PCMB is capable of inhibiting both the 2:4-dinitrophenol and the magnesium-activated mitochondrial ATPases[12, 25] it is possible that blockade of the ATP enzymic hydrolysis rather than the inhibition of some reaction occurring during oxidative phosphorylation may account for the ability of TET to block the ATP reversal of thyroxine induced swelling. It is interesting to note that TET inhibits ATPase activity at the same concentration (10^{-6} M) which blocks ATP reversal of thyroxine swelling; at this concentration TET has only minimal effects on oxidative phosphorylation.

ATP, and to a lesser extent ADP, reverse mitochondrial swelling induced by low concentrations of TET (10^{-7} M). At higher concentrations of TET these nucleotides cannot reverse the resultant swelling unless manganese ions are present. Both ATP and ADP, the latter probably by conversion to ATP via adenylate kinase, may activate some contractile process in the mitochondrial membrane. How the manganese ions markedly potentiate the actions of ATP is not presently known although this ion has been reported to have remarkable stabilizing actions on mitochondria.[3, 15]

The role of the electron transport chain on mitochondrial swelling has been studied by several investigators.[18–20] Hunter[18] proposed that substances that promote electron transport should promote swelling while substances inhibiting the transport of electrons along the chain should block swelling. Exceptions to this hypothesis are those agents which have a direct effect on the mitochondrial membrane. The present study does not conflict with this hypothesis since TET, which has no effect on electron transfer[26] is incapable of blocking glutamate or succinate-induced swelling. Furthermore, blockade of the electron transport chain by cyanide prevents TET-induced swelling.

It is difficult to assess the significance of TET-induced mitochondrial swelling in relation to the actions of this compound on other mitochondrial functions. Tapley[3] concluded that since thyroxine caused mitochondrial swelling at a concentration lower than that required to uncouple oxidative phosphorylation, the ability of thyroxine to alter mitochondrial membrane integrity was the primary action of the hormone on the mitochondrion. This might also be stated for the actions of TET since swelling is produced at a concentration of TET which is lower than that which effectively uncouples phosphorylation. However, since in the oxidative phosphorylation studies other factors (sucrose, ATP, ADP, Mg^{2+}, phosphate) are present which have pronounced actions on mitochondrial stability it is difficult to compare the relative effectiveness of TET on the two systems. The ability of TET to cause swelling probably explains the mitochondrial requirement for diphosphopyridine nucleotide which was observed during the oxidative phosphorylation studies.[12]

Although TET caused swelling of liver mitochondria *in vitro*, no swelling was observed in liver mitochondria from TET-treated rats. It should be emphasized that the medium in which swelling is measured *in vitro* is much different from that of the intact cell. Intracellular swelling is undoubtedly controlled by a dynamic balance of substrates, cofactors and ions so that marked mitochondrial swelling within the cell is probably produced only when an extreme imbalance of these factors occurs.[27] Presumably such an imbalance does occur in the brain during the course of TET toxicity since a recent study utilizing the electron microscope has shown that the glial cells and mitochondria in the brains of TET treated mice are swollen.[22] In the light of this finding it is paradoxical that no swelling is observed when brain mitochondria are treated *in vitro* with TET.

Although mitochondrial swelling has some properties in common with other membrane transport phenomena[15] it is difficult to assess the importance of this effect in relation to the actions of TET upon the whole animal. Some effects of TET that might be pertinent to the present study are the observations that this compound produces cerebral swelling[9, 21, 23] and inhibits water and glucose transport in the rat intestine.[28]

Acknowledgements—The technical assistance of Mr. John Fuscaldo in these studies is gratefully acknowledged. Triethyltin acetate was obtained from Mr. E. L. Weinberg, Metal and Thermit Company, Rahway, N.J. Rats used in this study were obtained from the Upjohn Company, Kalamazoo, Michigan through the courtesy of Dr. Burton Baker. These studies were aided by a contract between the office of Naval Research, Department of the Navy and the University of Michigan, NR 303-439.

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THE INFLUENCE OF ANTIMALARIAL DRUGS ON NUCLEIC ACID SYNTHESIS IN *PLASMODIUM GALLINACEUM* AND *PLASMODIUM BERGHEI*

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Abstract—The effect of antimalarial drugs upon uptake of P^{32}-labeled phosphate into ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) of chick blood infected with *Plasmodium gallinaceum* was studied *in vitro*, and was confirmed by experiments with *P. gallinaceum in vivo* and *P. berghei in vitro*. Quinine, chloroquine and quinacrine (Atabrine) inhibited the incorporation of ^{32}P into both RNA and DNA, whereas pyrimethamine and the triazine metabolite of chlorguanide specifically inhibited its incorporation into DNA. The inhibition by pyrimethamine was not reversed by folic acid, folinic acid, thymidine, deoxyuridine, uracil, thymine, glycine, or adenine. Chlorguanide was active *in vivo* but not *in vitro*. Pentaquine was inactive *in vivo* and *in vitro*.

INTRODUCTION

A SIGNIFICANT observation in the study of drug action on plasmodia was the finding of Clarke[2] that quinine at 10^{-5} M completely inhibited the incorporation of ^{32}P-labeled phosphate into deoxyribonucleic acid (DNA) of *Plasmodium gallinaceum*. This was especially pertinent because many of the previous studies were hampered in their interpretation by the high concentration of drug needed to obtain a biochemical effect.

The inhibition by pyrimethamine of the conversion of folic to folinic acid in bacteria[3] and liver[4] suggested that the antimalarial effect of pyrimethamine may also be due to an inhibition of nucleic acid synthesis.

It was of interest, therefore, to re-examine the effect of antimalarial drugs on the synthesis of nucleic acids in malarial parasites. In this report the uptake of ^{32}P by *Plasmodium gallinaceum in vitro* is investigated in detail, and confirmatory experiments with *P. gallinaceum in vivo* and *P. berghei in vitro* are reported. It is shown that quinine, chloroquine, and quinacrine (Atabrine) inhibit the incorporation of ^{32}P into both ribonucleic acid (RNA) and deoxyribonucleic acid (DNA), whereas pyrimethamine and the triazine metabolite† of chloroguanide[5, 6] specifically inhibit its incorporation

* Some of this work has been published as an abstract.[1]

† Formulas of the substances are as follows: pyrimethamine, 5-(*p*-chlorophenyl)-2:4-diamino-6-ethylpyrimidine; chlorguanide, N_1-*p*-chlorophenyl-N_5-*iso*propyl-biguanide; triazine metabolite of chlorguanide, 1-(*p*-chlorophenyl)-2:4-diamino-6:6-dimethyl-1:6-dihydro-1:3:5-triazine; DR15324, 6-hydroxy-8-(5-*iso*propylamino*iso*amylamino)-quinoline; M-266, 2-*cyclo*hexyl-3-hydroxy-1:4-naphthoquinone.

into DNA. The inhibition caused by pyrimethamine is not reversed by folic acid, folinic acid, thymidine, glycine, or other purine or pyrimidine bases. The 8-aminoquinolines are relatively inactive in all the systems studied, and chlorguanide is active only *in vivo*. The correlation of these observations with drug mechanisms is discussed.

EXPERIMENTAL

One-week-old New Hampshire Red chicks with from 60 to 80 per cent parasitized erythrocytes and with a reticulocyte count of less than 5 per cent were selected as donors for the *P. gallinaceum* experiments. The parasitized blood was obtained by heart puncture under sterile conditions with 0·2 vol. of 1 per cent heparin added as anticoagulant.

The incubations *in vitro* were carried out in rocker-dilution flasks described by Geiman *et al.*[7] Each flask contained 0·5 ml parasitized blood in 4·5 ml of a medium containing 0·5 μc ^{32}P-labeled inorganic orthophosphate with specific activity of 40 mc/mg, 400 units potassium penicillin G, 0·071 M NaCl, 0·039 M KCl, 0·024 M $KHCO_3$, 0·001 M $MgCl_2$, and 0·016 M glucose, with drug in a final concentration as noted. The flasks were rocked at 17 cyc/min inside an incubator maintained at 37 °C. A water-saturated gas mixture containing 7 per cent CO_2 and 93 per cent air was slowly passed over the flask contents. Incubation time was 22 hr unless otherwise noted. During incubation the parasitized red cells tended to form sticky masses which adhered to the bottom of the flasks. These were removed with a wire loop. Blood agar cultures taken of all flasks were usually negative: positive ones were discarded.

The nucleic acids were isolated according to a slight modification of procedure II of Davidson and Smellie,[8] since early experiments revealed that the Schmidt–Thannhauser method[9] failed to give DNA free from radioactive contamination by labeled RNA or other phosphate-containing substances. In the Schmidt–Thannhauser procedure, the acid-soluble phosphorus was extracted with cold 5 per cent trichloroacetic acid, and the lipid phosphorus with ethanol and 3:1 ethanol–ether. RNA was then hydrolyzed by treatment with 0·3 M KOH for 18 hr at 37 °C. DNA was precipitated, and the products of hydrolysis of RNA were extracted with cold 5 per cent trichloroacetic acid. DNA was then extracted from the remaining protein with hot 5 per cent trichloroacetic acid. The Davidson and Smellie procedure involved the following changes: after extraction of acid-soluble and lipid-phosphorus as noted above, the RNA and DNA were extracted with 10 per cent NaCl for 1 hr at 100 °C. The low yield of RNA in this procedure was shown to be due to hydrolysis in the course of extraction with hot NaCl. The nucleic acids were precipitated by the addition of ethanol, and were washed with cold trichloroacetic acid. The RNA was then separated from DNA by alkaline hydrolysis as in the Schmidt–Thannhauser procedure. The DNA remaining after hydrolysis of RNA required no further separation. Acid-soluble and lipid extracts were analyzed for total phosphate[10] and RNA and DNA fractions were analyzed for ribose,[11] deoxyribose,[12] and total phosphate. Aliquots of all fractions were dried on steel planchets and radioactivity was measured in a proportional counter with a counting efficiency of 45 per cent. Counting error for all samples was no more than 3 per cent. Suitable corrections were made for decay and background.

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Chemicals were of reagent grade or highest purity obtainable. We are grateful to Dr. Louis F. Fieser for supplying samples of lapinone and M-266.

RESULTS

The nucleic acids extractable from normal and parasitized chick blood by both procedures are shown in Table 1. The hematocrit averaged 25 per cent and 19 per cent, red count $1{\cdot}82 \times 10^6$ and $1{\cdot}24 \times 10^6$ red cells/μl blood, with mean corpuscular volume of 126×10^{-9} and 157×10^{-9} μl/red cell for normal and parasitized blood, respectively. The DNA was then $2{\cdot}22 \times 10^{-6}$ and $2{\cdot}62 \times 10^{-6}$ μg/cell for normal and parasitized blood, respectively. Lewert[13] obtained the value of $2{\cdot}24 \times 10^{-6}$ μg/cell for normal blood.

TABLE 1. PHOSPHORUS COMPONENTS IN NORMAL CHICKEN BLOOD AND CHICKEN BLOOD PARASITIZED WITH *P. gallinaceum*

Fraction	mg P/ml packed RBC*	
	Normal blood	Parasitized blood†
Total phosphorus	4·37	5·20
Acid-soluble phosphorus	1·36	1·45
Lipid phosphorus	0·43	0·69
RNA‡	0·26	0·81
RNA§	0·03	0·13
DNA‡	1·76	1·67
DNA§	0·77	0·94
Residue‡	0·50	0·38

* Values are averages of two or more experiments. The standard error of the mean[17] was usually about 10 per cent of the value.

† Donors were bled during the phase of increasing parasitemia, with from 60 to 80 per cent of their erythrocytes parasitized, and with less than 5 per cent reticulocytes.

‡ Fractionated according to Schmidt and Thannhauser.[9]

§ Fractionated according to procedure II of Davidson and Smellie.[8]

By either procedure the RNA contained an average of 7·7 per cent and DNA 7·5 per cent, phosphorus. Analysis of both RNA and DNA for ribose and deoxyribose indicated that neither nucleic acid was detectably contaminated with the other.

Experiments relating the incorporation of labeled phosphate to incubation time are shown in Table 2. The incorporation was calculated as the specific activity in counts/min per mg P of the fraction (lipid, RNA, DNA) divided by the counts/min per mg P of the acid-soluble phosphate, and expressed as μg P incorporated/mg fraction P. The specific activity of the acid-soluble fraction remained constant during the incubation; in fifteen experiments the average values were 5900 and 5800 counts/min per mg P at 0 and 22 hr, respectively. Analysis of the acid-soluble fraction after incubation revealed that 94 per cent of the radioactivity remained in the form of inorganic phosphate. Thus, in confirmation of previous studies,[14–16] the acid-soluble phosphate behaved as a homogeneous pool from which the other fractions were derived. In the total of thirty-seven experiments the 22-hr mean values $\pm$s.e.m.[17] for

phosphorus incorporation into RNA and DNA were 158 ± 17 and 7·7 ± 0·8 μg/mg, respectively. The incorporation into lipid phosphate was not routinely determined, since several experiments indicated that this was not appreciably affected by antimalarial drugs. Normal blood (Table 2) showed no incorporation of phosophate into DNA, and only about 6 per cent of the incorporation into RNA that was exhibited by parasitized blood. It should be noted that the parasite DNA is a small fraction of the total cell DNA, which is mainly contributed by the red cell nucleus. The actual specific activity is the average of the highly active incorporation into parasite DNA and

TABLE 2. INCORPORATION OF ^{32}P-PHOSPHATE INTO COMPONENTS OF *Plasmodium gallinaceum*

Exp.	Time incubated (hr)	Incorporation, (μg/mg)		
		Lipid	RNA	DNA
1	0	1·2	0·4	0·0
	3	2·8	3·5	0·1
	6	6·7	10·5	0·3
	12	15·2	23·4	1·2
	24	42	54	7·2
2	0	—	3·4	0·0
	7·6	—	47·5	0·7
	24	—	239	5·1
3 (Non-infected blood)	24	—	10·1	0·0

Parasitized blood was incubated at 37 °C in rocker-dilution flasks. The incorporation of phosphorus into lipid, RNA and DNA was calculated from the relative specific activity of these substances as compared with the acid-soluble fraction.

the inert larger amount of red cell DNA. In a species with non-nucleated red cells (*Plasmodium berghei* in mice), direct measurement of parasite DNA[18] gives a value of $0{\cdot}59 \times 10^{-7}$ μg/parasite, or about 2 per cent of the amount of DNA per chick red cell.

Variations in incubation media revealed that glycyl-glycine would not substitute for bicarbonate as buffer, although the pH was about 7·4 in both cases. Other inhibitory treatments were the use of 3 per cent bovine serum albumin, 10^{-3} M octanoate, and the washing of the infected blood free of serum. Little effect on phosphorus incorporation into RNA or DNA was noted by inclusion of 15 per cent red cell extract,[19, 20] 10 per cent normal chick red cells, 20 per cent unheated or heated (60 °C for 1 hr) chick serum, or by hemolysis of the infected red cells with rabbit serum.[21] Inclusion of the following vitamins was likewise without effect: B_{12}, folic acid, biotin, α-lipoic acid and *p*-aminobenzoic acid 10^{-8} M; riboflavin, thiamine, pantothenate, pyridoxine, choline and inositol 10^{-6} M; ascorbic acid and nicotinamide 10^{-5} M. Media with sodium as the principal cation completely replacing potassium gave results the same as the control medium. Plasma hydrolyzate 10 per cent (Travamin), folic acid from 10^{-8} to 10^{-5} M, folinic acid from 10^{-8} to 10^{-4} M, increase of phosphate to 10^{-3} and 10^{-2} M, glycine, thymidine, or adenine 10^{-4}, singly or in combination, thymine 10^{-5} M, or pyruvate 10^{-2} M plus malate 10^{-3} M also gave incorporations similar to that in the standard medium.

Pyrimethamine, at a level as low as 10^{-8} M, caused marked inhibition of P^{32}-incorporation into DNA, with no effect on RNA. In Fig. 1 the effect of pyrimethamine is expressed as the average incorporation in all the experiments with various concentrations of the drug. Attempts to prevent the action of pyrimethamine 10^{-7} M by inclusion, at 10^{-5} and 10^{-4} M, of thymidine, thymine, thymidylate, deoxyuridine, uracil, folic acid, folinic acid, glycine, adenine, or a combination of glycine, adenine, and thymidine,[22] were all without success.

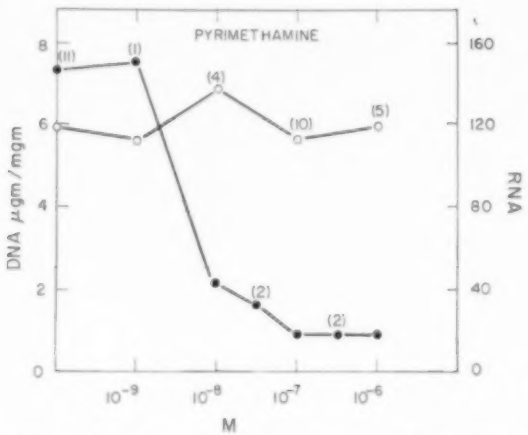
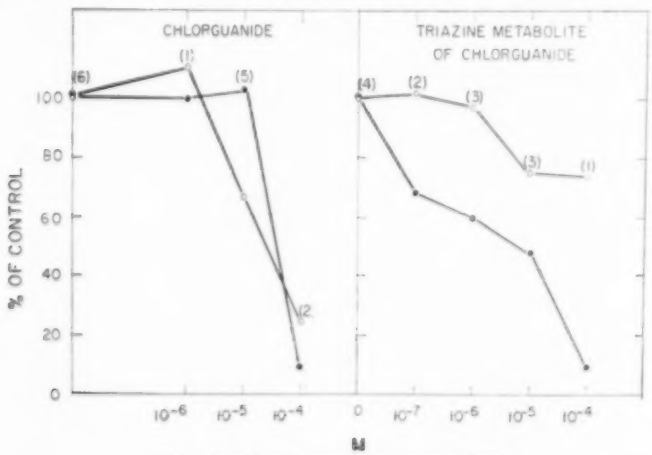


FIG. 1. Inhibition of DNA synthesis by pyrimethamine. *In vitro*-incubation of *P. gallinaceum*. Incorporation of P in μg/mg. ○ = RNA, ● = DNA. Abscissa, drug concentration. Each point represents the average value obtained from the number of experiments shown in parentheses.

The effect of other antimalarial drugs is shown in Figs. 2 to 6. Only the triazine metabolite of chloroguanide specifically inhibited DNA synthesis, whereas chlorguanide was inactive and only at 10^{-4} M did it cause a nonspecific inhibition of both RNA and DNA incorporation. Quinine, chloroquine, and quinacrine, at about 10^{-5} M, caused an inhibition of the incorporation of phosphate into both RNA and DNA.



FIGS. 2 and 3. See caption on page 148

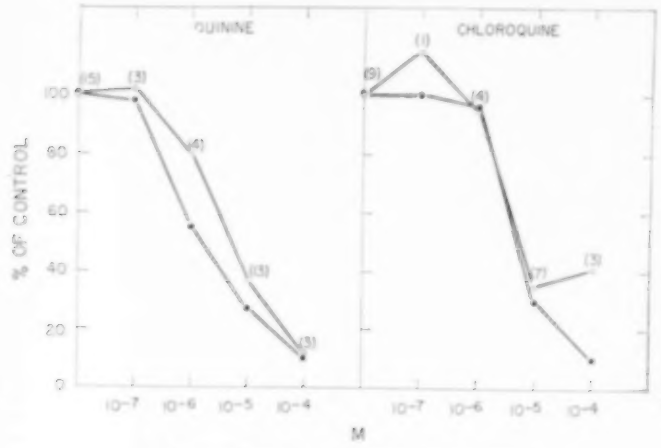


Fig. 4

Fig. 5

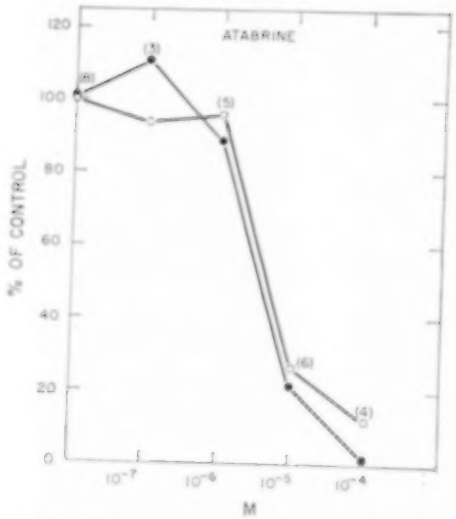


Fig. 6

FIGS. 2–6. Inhibition of P^{32}-phosphate incorporation into nucleic acids by anti-malarials. *In vitro* incubation of *P. gallinaceum*. Results shown as a percentage of control for each experiment vs. drug concentration. ○ = RNA, ● = DNA, otherwise same as Fig. 1.

The other cinchona alkaloids acted similarly to quinine (Table 3). The 8-aminoquinolines and a 6-hydroxy analog of pentaquine (DR 15324) were relatively inactive. Of the antibiotics only puromycin was markedly inhibitory at 10^{-5} M. Aminopterin, amethopterin and sulfathiazole were inactive.

The inhibition of the incorporation of ^{32}P into DNA by antimalarial drugs was confirmed *in vivo* (Fig. 7). Chicks weighing from 65 to 137 g, with from 5 to 10 per cent parasitemia, were given 2 μc of ^{32}P-phosphate intraperitoneally and drug intraperitoneally or orally at 0 and again at 12 hr. At 24 hr, 2 ml of heart blood was processed by procedure II of Davidson and Smellie,[8] and the specific activity of acid-soluble, lipid, RNA, and DNA determined. Drug dosages in mg/kg $\pm$ s.e.m.[17] were:

quinine 400 ± 20, chloroquine 23 ± 2, pentaquine 4·8 ± 0·4, chlorguanide 42 ± 7, triazine metabolite 34, and pyrimethamine 1·5 ± 0·2. The incorporation of ^{32}P is calculated in the same manner as in the *in vitro*-experiments. The specific activity of the acid-soluble, lipid, and RNA phosphorus was identical, indicating equilibration of the label among these components. DNA had not reached this equilibrium; the graph

TABLE 3. THE EFFECT OF VARIOUS INHIBITORS ON NUCLEIC ACID INCORPORATION OF P^{32}-PHOSPHATE IN *Plasmodium gallinaceum*

Substance	Concentration (M)	No. exp.	Percentage of control	
			RNA	DNA
A. Antimalarials				
Quinidine	10^{-5}	1	35	14
cinchonine	10^{-5}	2	28	20
cinchonidine	10^{-5}	2	18	14
primaquine	10^{-5}	2	90	110
primaquine	10^{-4}	1	5	0
pentaquine	10^{-5}	6	56	65
pentaquine	10^{-4}	1	45	13
DR 15324	10^{-5}	6	43	50
DR 15324	10^{-4}	1	35	5
lapinone	10^{-7}	2	116	124
lapinone	10^{-6}	2	95	125
lapinone	10^{-5}	2	100	136
lapinone	10^{-4}	1	5	0
M 266	10^{-7}	2	92	121
M 266	10^{-6}	1	133	100
M 266	10^{-5}	1	70	92
B. Antibiotics				
tetracycline	10^{-5}	1	90	112
tetracycline	10^{-4}	1	50	6
chloromycetin	10^{-4}	2	101	96
erythromycin	10^{-5}	1	133	64
neomycin sulfate	6 mg/l	1	132	67
puromycin	10^{-5}	2	40	11
stilbamidine	10^{-5}	1	127	66
fumagillin	10^{-5}	1	94	106
C. Folic acid antagonists				
aminopterin	10^{-6}	1	112	109
aminopterin	10^{-5}	3	91	104
amethopterin	10^{-6}	2	83	78
amethopterin	10^{-5}	2	108	58
sulfathiazole	6×10^{-4}	4	104	101
D. Others				
colchicine	10^{-5}	1	94	94
pentachlorophenol	10^{-5}	2	105	90
pentachlorophenol	10^{-4}	1	0	0
nikethamide	10^{-4}	1	104	30
ethionine	10^{-4}	1	104	113

Incubated as described in text. ^{32}P-incorporation reported as a percentage of control without inhibitor.

depicts the specific activity of DNA as a fraction of that of the other components. Each bar represents one chick. Here therapeutic doses of quinine, chloroquine, chlorguanide, triazine metabolite of chlorguanide, and pyrimethamine, but not pentaquine, inhibited the incorporation into DNA of the parasitized blood.

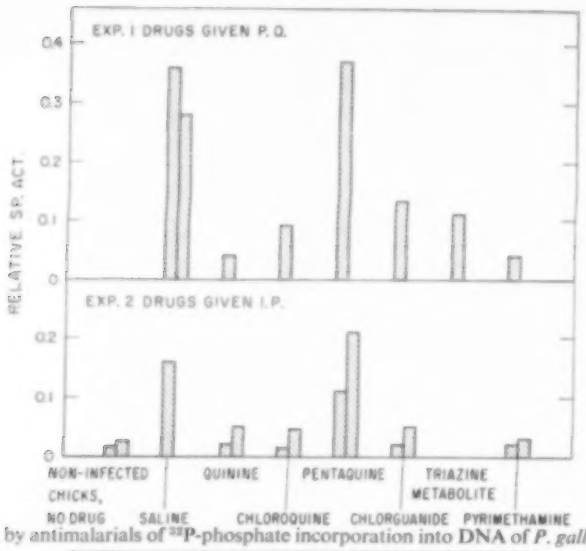


FIG. 7. Inhibition by antimalarials of ^{32}P-phosphate incorporation into DNA of *P. gallinaccum in vivo*.

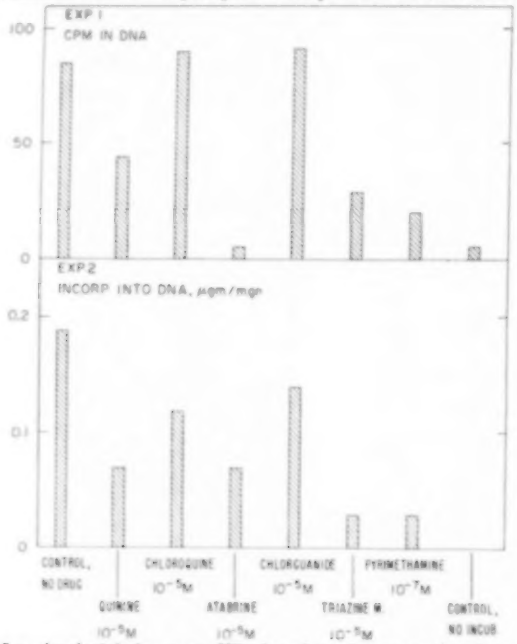


FIG. 8. The effect of antimalarial drugs on ^{32}P-phosphate incorporation into DNA of rat blood infected with *Plasmodium berghei*. Heparinized blood from young rats infected with *P. berghei* was collected by heart puncture when parasitemia was 40 and 52 per cent, respectively, in the two experiments. The incubations were carried out as described in the text. The acid-soluble and DNA phosphate were isolated as in the chick experiments. In experiment 1, the DNA phosphate content of the preparations was too low to determine, hence the radioactivity of only the DNA is reported. In experiment 2, 0·5 ml of non-parasitized chicken blood was added to each flask at the end of the incubation to provide carrier DNA. The result here is reported as mg ^{32}P/mg DNA P, as in the chick experiments. Each bar represents one flask.

The results of two experiments *in vitro* with *P. berghei* are shown in Fig. 8. The drugs exhibited similar behaviour in the two species with the curious exception of chloroquine, which showed little or no inhibition of incorporation of ^{32}P by *P. berghei*.

DISCUSSION

The effect of pyrimethamine and chloroguanide through its metabolite appear to be the most pertinent, since these compounds specifically inhibit the incorporation of ^{32}P-phosphate into DNA in all the systems studied. The present observations are consonant with prior studies which indicate that these drugs primarily inhibit multiplication without affecting viability. Clinically, the drugs act slowly and appear to attack only the dividing forms of plasmodia.[23–25] Pyrimethamine is effective only against the actively growing, rather than the dormant, stages of toxoplasmosis.[26] Viability of *P. gallinaceum*, as measured by infectivity, was not affected by pyrimethamine *in vitro*.[27] The parasites thus appear to remain viable despite inhibition of cell multiplication. Pyrimethamine is known to inhibit the transformation of folic acid to folinic acid.[3, 4] If such an inhibition caused the blocking of DNA synthesis in our experiments, then folinic acid should have reversed the inhibition. Folinic acid apparently is utilized by the free parasite, since Trager[28] observed that the survival *in vitro* of *P. lophurae* outside the red cell was enhanced by folinic acid. The inability of folinic acid to reverse the inhibition by pyrimethamine here suggests either non-utilization of added folinic acid by the parasite when it is within the red cell, or an additional interference of DNA synthesis by pyrimethamine at a point beyond folinic acid.

Chlorguanide was shown to be effective only *in vivo*, whereas its triazine metabolite was also active *in vitro* (Figs. 2, 3, and 7). Evidently the metabolite is the active form of the drug,[5, 6] and was formed by the host *in vivo*. Pyrimethamine and the triazine metabolite of chlorguanide have been postulated to have similar modes of action[29, 30] on the basis of structural similarity, mutal cross-resistance,[30, 31] similar clinical effect,[31] potentiation by sulfonamides,[30, 32] and reversal by folic acid derivates.[30, 33]

The inhibition of the incorporation of ^{32}P-phosphate into RNA and DNA by the cinchona alkaloids, chloroquine, and quinacrine is probably related more closely to inhibition of substrate utilization[34, 35] than to nucleic acid synthesis.

The relative inactivity of the 8-aminoquinolines (pentaquine, primaquine, and DR 15324), *in vitro* and *in vivo*, indicates that these drugs have a mode of action unrelated to any effect on phosphate assimilation into RNA or DNA. Since the 8-aminoquinolines have been shown to form active metabolites *in vivo*,[27, 36, 37] any active metabolite formed was likewise inactive.

A number of substances (lapinone, 8-aminoquinolines, tetracycline, pentachlorophenol) were inactive at 10^{-5} M, but markedly inhibitory at 10^{-4} M for both RNA and DNA incorporation (Table 3). This may denote an interference with energy utilization by the parasite. The naphthoquinones are known to inhibit electron transport[38, 39] and pentachlorophenol uncouples oxidative phosphorylation.[40] The results with the other substances (Table 3) generally correlated with their lack of antimalarial activity. The antibiotics were inactive as expected,[41] except for puromycin. The inactivity of the sulfonamide here, and its known slow action against plasmodia *in vivo*,[42] may reflect an inhibition of folinic acid synthesis, the endogenous supply sustaining growth during the experimental period. Similarly, aminopterin and amethopterin may be inactive in

this system and inactive as antimalarials because of utilization of endogenous folinic acid by the parasite or because the red cell is impermeable to this substance. It may be recalled here that duck blood parasitized with *P. lophurae* has been shown to contain from ten to twenty times as much folinic acid as normal blood.[43]

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A COMPARISON OF THE ACTION OF TRIETHYLTIN WITH OTHER DRUGS ON CREATINE PHOSPHATE LEVELS IN RAT BRAIN AND DIAPHRAGM PREPARATIONS

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Abstract—The effect of triethyltin sulphate on the oxygen consumption and creatine phosphate synthesis of brain slices has been measured. The results have been compared with those obtained using chlorpromazine, dinitrophenol (DNP) and pentachlorophenol. In the presence of triethyltin and chlorpromazine, creatine phosphate synthesis was inhibited to a greater extent than oxygen uptake and creatine leaked from the slice into the medium. DNP and pentachlorophenol stimulated oxygen uptake and inhibited creatine phosphate synthesis, but did not cause a leakage of creatine from the slice. Identical experiments using diaphragm segments gave essentially similar results except for triethyltin which was without effect.

Experiments using a phrenic-nerve diaphragm preparation confirmed that in the presence of DNP and pentachlorophenol there is a failure in the response to electrical stimuli and a depletion of creatine phosphate in the diaphragm. In the presence of triethyltin the preparation also failed to respond normally to electrical stimuli but the creatine phosphate of the diaphragm was not lowered.

DURING the past ten years a wide variety of drugs and poisons have been tested for their ability to uncouple oxidative phosphorylations. Such tests have usually been made on isolated mitochondrial preparations, particularly from rat liver. The positive findings have been used in many instances to help explain the *in vivo* effects even though the response of animals to the different drugs often varied widely in character.

Several of the compounds examined appear to have a selective action *in vivo* on central nervous tissue; but when tested *in vitro* against liver mitochondrial preparations they are also active.[1, 2, 3]

McIlwain and co-workers[4] have shown that it is possible to measure the synthesis of creatine phosphate in cerebral cortex slices incubated in a saline medium containing glucose. The action of several drugs on such preparations has already been examined.[5, 6] In a similar manner it is possible to measure the creatine phosphate content of incubated segments of rat diaphragm, and a few studies of drug action have been made on this preparation.[7, 8]

In the present work a direct comparison has been made between the action of triethyltin sulphate, chlorpromazine, dinitrophenol and pentachlorophenol on the levels of creatine and creatine phosphate in rat cerebral cortex slices and segments of rat diaphragm. In this way it has been possible to compare the action of these compounds on nervous and non-nervous tissue under virtually identical conditions.

A few experiments have also been made using a rat phrenic nerve-diaphragm preparation.

METHODS

Adult male albino rats weighing about 200 g were used. Brain cortex slices were prepared and used as previously described.[9] Segments of diaphragm approximately 7 × 7 mm were cut with scissors. For experiments with diaphragm the conditions were identical to those used for brain slices except that the concentration of Ca^{++} in the incubation medium was lowered by a factor of 3 to give a final concentration of 0·001 M. Higher and more consistent values for creatine phosphate were obtained using this modified Krebs–Ringer phosphate medium, and the concentration of Ca^{++} approximated more closely that of Tyrode's solution used for the phrenic nerve–diaphragm experiments.

At the end of the incubation period the pieces of tissue were dropped quickly into 4·0 ml of ice-cold 10% (w/v) trichloroacetic acid, homogenized and centrifuged at −5 °C. Free and total creatine were determined on aliquots of the supernatant by the colorimetric method of Eggleton, Elsden and Gough[10] as used by Ennor and Rosenberg[11]. The difference between the two was taken as a measure of creatine phosphate. After removing the tissue from the Warburg flasks 1·0 ml of 30% (w/v) trichloroacetic acid was added to the incubation fluid and aliquots were taken for total creatine estimations.

The isolated rat phrenic nerve–diaphragm preparation was that of Bülbring[12]. It was suspended in a bath of 60 ml capacity containing Tyrode's solution[13] at 37 °C and aerated with a mixture of 95 per cent O_2 and 5 per cent CO_2. Stimulation was by means of rectangular impulses of 200 μsec duration. The routine procedure adopted was as follows: The preparation was mounted in position and left for 30 min while the nerve was stimulated once every 5 sec. This was followed by a series of tetanic stimulations of 50, 100 and 200/sec for 5 sec duration at 1 min intervals. A dose of the inhibitor was then added to the bath and a series of tetanic stimulations applied at 15 and 30 min. Exactly 5 min after the last tetanus, the preparation was plunged into 10% trichloroacetic acid at −5 °C, homogenized, centrifuged, and aliquots of supernatant taken for free and total creatine estimations as described above.

Lactic acid was estimated by the technique of Barker and Summerson[14] and pyruvic acid by the method of Friedemann and Haugen[15] with the modification for increasing the sensitivity previously described.[16] Triethyltin was estimated as described earlier.[9]

RESULTS

During the preparation of slices of brain cortex it has already been shown,[4] and confirmed by us, that the creatine phosphate content falls to low or zero levels. On incubation in a suitable medium containing glucose, creatine phosphate is re-synthesized reaching a fairly constant level of 1·6 μmoles/g. In Table 1 the effect of triethyltin sulphate and chlorpromazine hydrochloride together with those of dinitrophenol and pentachlorophenol (classical uncouplers of oxidative phosphorylation), on oxygen consumption, free and total creatine levels in brain slices is given. With low concentrations of triethyltin the oxygen consumption was slightly stimulated while the level of creatine phosphate was lowered. At concentrations of triethyltin which inhibited oxygen consumption between 15 and 55 per cent creatine phosphate synthesis was inhibited between 90 and 100 per cent, and at the same time there was a leakage of creatine from the slice into the medium. Chlorpromazine produced similar effects, although the difference between the percentage inhibition of oxygen consumption

and creatine phosphate synthesis was less marked, but the leakage of creatine from the slice to the medium was greater than in the presence of triethyltin. Dinitrophenol and pentachlorophenol on the other hand stimulated the consumption of oxygen and inhibited the synthesis of creatine phosphate. Even when creatine phosphate synthesis was completely inhibited creatine did not leak into the medium.

TABLE 1. EFFECT OF TRIETHYLTIN AND OTHER COMPOUNDS ON CREATINE LEVELS IN BRAIN SLICES

Compound	Concentration (M)	Tissue				Medium
		O_2 uptake	Free Creatine	Total Creatine	Creatine phosphate	Total Creatine
—	—	2·09–2·21(4)	3·49–4·76(4)	4·97–6·45(4)	1·48–1·69(4)	5·17–5·96(4)
Triethyltin sulphate	$2{\cdot}62 \times 10^{-7}$	2·38	4·46	5·53	1·06	5·61
	$3{\cdot}65 \times 10^{-7}$	1·86	4·4	4·46	0·06	6·67
	$5{\cdot}5 \times 10^{-7}$	1·26	4·0	3·89	0	7·36
Chlorpromazine-hydrochloride	$9{\cdot}7 \times 10^{-5}$	2·09	4·42	5·92	1·5	7·0
	$2{\cdot}9 \times 10^{-4}$	1·5	2·21	2·85	0·64	10·75
Dinitrophenol	$1{\cdot}1 \times 10^{-5}$	2·89	4·79	5·34	0·55	5·72
Pentachlorophenol	$2{\cdot}5 \times 10^{-6}$	3·46	6·34	6·18	0	5·92

Each flask contained 3 ml of Krebs–Ringer phosphate with 0·011 M glucose, triethyltin or other compounds as indicated, tissue slice of 50–60 mg wet wt and 0·2 ml of 20% (w/v) KOH in the centre well for absorption of CO_2. The manometers were placed in a bath at 37°, gassed for 5 min with 100% O_2, equilibrated for a further 10 min and O_2 uptake was measured at intervals up to 75 min. Results are the arithmethical mean of duplicate experiments except for the controls where the range of individual values are given followed by the number of experiments in parenthesis. O_2 uptake is expressed as μl of O_2/mg wet wt tissue per hr and creatine as μmoles/g wet wt tissue.

TABLE 2. EFFECT OF TRIETHYLTIN AND OTHER COMPOUNDS ON CREATINE LEVELS IN DIAPHRAGM SEGMENTS

Compound	Concentration (M)	Tissue				Medium
		O_2 uptake	Free Creatine	Total Creatine	Creatine phosphate	Total Creatine
—	—	0·9–1·25(5)	5·07–6·81(5)	14·02–17·25(5)	8·6–10·95(5)	10·71–13·7(5)
Triethyltin sulphate	$4{\cdot}9 \times 10^{-6}$	1·2	8·09	14·1	6·01	11·73
	$7{\cdot}9 \times 10^{-6}$	1·18	6·37	14·51	8·14	14·3
Chlorpromazine-hydrochloride	$1{\cdot}9 \times 10^{-4}$	1·39	6·44	10·81	4·37	16·7
	$3{\cdot}2 \times 10^{-4}$	1·16	3·72	3·45	0	21·1
Dinitrophenol	$5{\cdot}6 \times 10^{-6}$	1·65	8·5	11·28	2·68	11·06
	$1{\cdot}1 \times 10^{-5}$	1·87	9·3	7·52	0	13·8
Pentachlorophenol	$8{\cdot}3 \times 10^{-7}$	1·81	7·26	11·39	4·13	10·0
	$1{\cdot}05 \times 10^{-6}$	1·9	13·11	16·76	3·65	12·4

For details see Methods and Table 1.

Similar experiments were carried out using cut segments of rat diaphragm. Unlike cerebral cortex slices, diaphragm segments only lose a small amount of creatine phosphate during the preparative procedure. The level of creatine phosphate at the time of

placing the tissue in the flasks was about 85 per cent of that found after incubation for 75 min. The action of the four drugs on diaphragm segments is shown in Table 2. Triethyltin, even at high concentrations compared with those active against brain slices, had little or no effect on oxygen consumption or creatine phosphate levels.

TABLE 3. DISTRIBUTION OF TRIETHYLTIN BETWEEN TISSUE AND MEDIUM WITH BRAIN AND DIAPHRAGM

Tissue	Concentration (M) of triethyltin		R
	Added in medium	Found in tissue	
Brain slice	$5{\cdot}3 \times 10^{-5}$	$10{\cdot}4 \times 10^{-4}$	20
	$1{\cdot}8 \times 10^{-5}$	$2{\cdot}7 \times 10^{-4}$	15
Diaphragm segment	$4{\cdot}9 \times 10^{-5}$	$7{\cdot}6 \times 10^{-4}$	16
	$1{\cdot}6 \times 10^{-5}$	$2{\cdot}9 \times 10^{-4}$	18

The amount of triethyltin found in the tissue after 75 min incubation was calculated as the molar concentration in the tissue fluid taking the tissue fluid as 80 per cent of the wet weight.

R is the quotient: molar concentration of triethyltin in tissue fluid/molar concentration added in medium.

TABLE 4. CREATINE LEVELS IN BRAIN SLICES AND DIAPHRAGM SEGMENTS FROM RATS INJECTED WITH TRIETHYLTIN SULPHATE

Tissue	Animal	Tissue				Medium
		O_2	Free creatine	Total creatine	Creatine phosphate	Total creatine
Brain slice	Control	2·15	4·24	5·85	1·61	5·38
	Injected	1·34	3·72	3·85	0·13	6·94
Diaphragm segment	Control	1·11	5·91	15·77	9·87	11·8
	Injected	1·43	8·08	17·6	9·52	12·3

Conditions were identical to those described in Tables 1 and 2.

Since earlier work[9] had shown that after incubating brain slices in a medium containing triethyltin the concentration found in the slice was 17 times greater than that in the medium at the beginning of the experiment, it was possible that the lack of effect of triethyltin on diaphragm segments was due to a lower penetration of the drug into this tissue. However, values given in Table 3 show that equal amounts of triethyltin entered diaphragm segments as entered brain cortex slices.

Chlorpromazine did not inhibit the oxygen consumption of diaphragm segments but lowered the creatine phosphate level, and, as with brain slices, caused a marked increase in the amount of creatine in the medium. Dinitrophenol and pentachlorophenol affected diaphragm segments in the same way as they affected brain slices at similar concentrations.

It has already been demonstrated that it is possible to show an altered carbohydrate metabolism in brain slices prepared from rats injected with triethyltin.[9] Measurements were therefore made of the oxygen consumption and creatine levels in brain slices and diaphragm segments prepared from animals killed 2 hr after an

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intraperitoneal injection of triethyltin sulphate 10 mg/kg. The results given in Table 4 show that brain slices were affected in the same way as when triethyltin was added *in vitro*, whereas diaphragm segments did not vary from the controls.

A few experiments were carried out using phrenic-nerve-diaphragm preparations. The procedure adopted is fully described under Methods. In the presence of the highest concentration of triethyltin used the preparation failed to respond normally to electrical stimulation but the level of creatine phosphate was not lowered (Table 5).

TABLE 5. EFFECT OF TRIETHYLTIN AND PENTACHLOROPHENOL ON RESPONSE TO STIMULI AND CREATINE LEVELS OF PHRENIC-NERVE DIAPHRAGM PREPARATION

Compound	Concentration (M)	Response to stimuli	Creatine phosphate μmoles/g
—	—		8·5
Triethyltin sulphate	$2·5 \times 10^{-6}$	Failure to hold a tetanus of 100 and 200 p.s.	6·8
	5×10^{-6}	Failure to hold a tetanus of 100 and 200 p.s.	8·58
	1×10^{-5}	Failure to hold a tetanus of 100 and 200 p.s. and reduced response to single stimuli	8·0
Pentachlorophenol	$1·88 \times 10^{-6}$	Contracture developed and a failure to hold a tetanus	0

For details see Methods.

TABLE 6. EFFECT OF TRIETHYLTIN AND OTHER COMPOUNDS ON AEROBIC AND ANAEROBIC GLYCOLYSIS OF BRAIN SLICES

Compound	Concentration (M)	Aerobic			Anaerobic
		Q_{O_2}	lactic acid μg/flask	pyruvic acid μg/flask	lactic acid μg/mg dry wt
—	—	12·4	186	11·4	46·3
Triethyltin sulphate	$1·3 \times 10^{-6}$	7·4	440	5·5	27·5
Chlorpromazine HCl	$2·4 \times 10^{-4}$	12·1	242	7·5	31·5
DNP	3×10^{-5}	—	—	—	45·0

Each flask contained 3 ml of Krebs–Ringer phosphate with 0·011 M glucose and triethyltin or other compounds as indicated. Average wet wt of tissue slice was 56 mg. The centre-well contained 0·2 ml of 20% (w/v) KOH. For aerobic experiments the gas phase was O_2 and for anaerobic experiments N_2 was used. After incubation for 75 min at 37° slices were removed and 3 ml of 18% (w/v) TCA was added. Samples of the flask contents were taken for lactic and pyruvic acid determinations. Q_{O_2} is expressed as μl of O_2/mg dry wt/hr, the aerobic lactic and pyruvic acids as μg of each acid/flask and anaerobic lactic acid as μg/mg dry wt of brain tissue.

After the addition of pentachlorophenol the preparation went into a state of contracture, failed to hold tetanic stimulations and creatine phosphate was depleted completely. These results were virtually identical to those of Barnes, Duff and Threlfall using dinitrophenol.[13] Addition of chlorpromazine hydrochloride at a concentration found to be effective in the Warburg experiments caused a white cloudy precipitate which interfered with the experiments.

DISCUSSION

As mentioned in the introduction a large number of studies have been devoted to testing the effect of drugs on the oxidative-phosphorylation capacity of mitchondrial preparations. All four compounds used in the present work have been shown to affect this process in rat liver mitochondria.[1, 2, 17] Although triethyltin and chlorpromazine inhibit both phosphate uptake and oxygen consumption in such preparations, the P : O ratio is lowered by both compounds. Dinitrophenol and pentachlorophenol increase oxygen consumption and decrease phosphate uptake. Fewer and sometimes conflicting results have been reported for similar experiments using brain mitochondria. This is probably due to the difficulties of preparing brain mitochondria which are metabolically stable.[18] Bernsohn, Namajuska and Cochrane[19] found that chlorpromazine inhibited oxygen and phosphate uptake of brain mitochondria, while Berger, Strecker and Waelsch[2] found that although chlorpromazine inhibited oxidative phosphorylation in liver mitochondria it was inactive against brain mitochondria. Dawkins, Judah and Rees[20], on the other hand, showed that there appeared to be no special difference between brain and liver mitochondrial preparations in their sensitivity to chlorpromazine.

The measurement of creatine phosphate in brain slices and diaphragm segments provides an alternative method for testing the effects of drugs on energy-rich phosphate compounds. The results with triethyltin sulphate show that it is an active inhibitor of creatine phosphate synthesis in brain slices (Table 1). Furthermore the synthesis of creatine phosphate appears to be more sensitive to triethyltin than oxygen consumption. In agreement with previous findings[9] triethyltin had virtually no effect on diaphragm segments. It could be shown that equal amounts of triethyltin entered both diaphragm and brain (Table 3). Thus triethyltin showed a selective action against metabolic processes of nervous tissue under the experimental conditions used. Chlorpromazine showed a definite inhibition of creatine phosphate synthesis in brain slices at a concentration of $2{\cdot}9 \times 10^{-4}$ M. Lindan, Quastel and Sved[21] showed that chlorpromazine inhibited the incorporation of glycine-^{14}C into proteins of brain slices at this concentration whereas oxygen consumption was less sensitive.

A striking difference between triethyltin and chlorpromazine became apparent when creatine levels of diaphragm segments were measured. Chlorpromazine lowered creatine phosphate at the same concentrations as it inhibited creatine phosphate synthesis in brain slices. It did not therefore share the selective effect on nervous tissue shown by triethyltin.

A common finding with triethyltin and chlorpromazine was that they both caused a leakage of creatine from brain slices into the medium. In a recent study of amino acid levels in brain slices,[22] it was shown that chlorpromazine caused an increase in the amount of glutamic acid and γ-amino-butyric acid appearing in the incubation fluid when glucose was added as substrate. The authors suggested that certain psychotropically active compounds tested by them, including chlorpromazine, may have two distinct modes of action, one on certain phases of the respiratory chain and the other on the membrane permeability. The present findings would lend support to such a view since in the presence of dinitrophenol and pentachlorophenol creatine phosphate levels in brain slices could be reduced to zero without there being an accompanying leakage of creatine into the membrane. Both compounds are known to act on the respiratory chain. The separate effects on membrane permeability may in some way

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help to explain the seemingly contradictory results on aerobic and anaerobic glycolysis of brain slices with triethyltin and chlorpromazine. Both compounds bring about an increase in lactic acid formation under aerobic conditions at concentrations which, under anaerobic conditions inhibit lactic acid formation (Table 6). Dinitrophenol did not inhibit anaerobic glycolysis of brain slices. It is highly probable that anaerobiosis is detrimental to the cells[23] so that the structural integrity of brain slices under anaerobic conditions may become more sensitive to certain agents. Neither chlorpromazine[19] nor triethyltin[16] inhibit anaerobic glycolysis of brain homogenates, which also suggests that the inhibition seen in brain slices is related to some structural alteration only apparent when whole-cell preparations are used.

Some pharmacological studies with triethyltin have already been reported.[24] Experiments using a phrenic-nerve diaphragm preparation showed that triethyltin at $2{\cdot}5 \times 10^{-6}$ M brought about a failure to hold a tetanus and at higher concentrations a reduced response to single stimuli. The response of the diaphragm to direct stimuli was not affected by triethyltin at 1×10^{-5} M.[24] The level of creatine phosphate was not lowered in the nerve-diaphragm preparation even after it failed to respond to stimuli in the presence of triethyltin. The results are quite dissimilar to those with DNP[13] or with pentachlorophenol (Table 6) where failure to respond to stimuli is accompanied by a large fall in creatine phosphate. Although no measurements have been made of the metabolism of isolated nerves in the presence of triethyltin it is possible that the phrenic nerve is sensitive to triethyltin since it has been shown that glucose metabolism by brain cortical grey and white matter and pieces of spinal cord are all inhibited in a similar manner.[9]

During the course of studies carried out in these laboratories on the mechanism of triethyltin intoxication attempts have been made to correlate the results of experiments carried out *in vitro* with changes occurring in the whole animal. Some of the difficulties arising have already been pointed out.[25] There seems little doubt that the predominant features of triethyltin poisoning are indicative of a central nervous effect.[26] Considerable progress towards understanding the production of an oedematous lesion in the brain by triethyltin has been made by Parsons[27]. By using ^{24}Na he was able to show that the earliest change produced by triethyltin *in vivo* is a stagnation of the sodium pools of the cerebrospinal fluid and brain. It is suggested by the same author that triethyltin may inhibit the supply of energy necessary to maintain the mechanism which controls cerebral salt and water balance. From results of experiments with triethyltin *in vitro* using either brain mitochondria or brain slices this would seem a possibility. However, no lowering of creatine phosphate or adenosine triphosphate (ATP) was found in brains of rats injected with triethyltin sulphate.[25] Similarly chlorpromazine, well-known for its psychotic action, does not cause a lowering of ATP in brains of injected animals.[28] On the other hand when 3:5 dinitro-O-cresol (a compound which closely resembles the nitro- and halo-phenols) was administered to rats *in vivo* it caused a marked decrease in the creatine phosphate and ATP content of brain.[29] ATP was also shown to be lowered in heart and diaphragm muscle but creatine phosphate values are not given.[29]

It would seem that a close agreement exists between the known effects of certain substituted phenol compounds on enzyme systems *in vitro* and their effect on the metabolism of animals *in vivo*. With triethyltin and chlorpromazine many discrepancies still exist between the results of studies made *in vivo* and *in vitro* and it is as

yet impossible to explain the predominance of central nervous effects by these two compounds in the living animal.

Acknowledgements—My thanks are due to Mr. D. Potter for valuable technical assistance and to Mr. W. R. Lewis of the Tin Research Institute for supplying triethyltin sulphate.

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EFFECT OF 5-BIS(2-CHLOROETHYL)AMINOURACIL ON INCORPORATION OF URACIL-2-^{14}C INTO RNA OF TUMOUR-BEARING RATS

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Abstract—At various time intervals following the administration, to rats bearing the Walker 256 carcinosarcoma, of 5-bis(2-chloroethyl)aminouracil in doses of 4 mg/kg, 20 μc of uracil-2-^{14}C were injected. The animals were sacrificed after 75 min, the tissues were excised, and the specific activities of the RNA of the intracellular fractions were determined.

The incorporation of uracil-2-^{14}C into the RNA of the various intracellular fractions of tumor and spleen was markedly inhibited. The effect on the spleen was more marked than was the effect on the tumor. In the liver, an increased incorporation of the labeled uracil into the RNA of all intracellular fractions was observed at 12 hr after the administration of the drug.

RECENT experiments carried out in this laboratory have indicated the extensive utilization of amino acids for the biosynthesis of nuclear proteins in transplantable rat tumors.[1] Other studies have demonstrated the inhibitory effect of 5-bis(2-chloroethyl) aminouracil, a mustard linked to a pyrimidine analog,[2] on the incorporation of L-arginine-U-^{14}C into nuclear proteins.[3] Kinetic studies revealed that a dose of 4 mg of the drug per kg suppressed the incorporation of L-arginine-U-^{14}C into the acid-soluble nuclear proteins within 6 hr after administration of the drug. The uptake of labeled arginine into the acid-soluble nuclear proteins was markedly inhibited after a lag period of 12 hr. The aminouracil mustard inhibited the incorporation of L-arginine-U-^{14}C into the nuclear proteins of this tumor before any impairment of growth could be detected.

Inasmuch as recent evidence indicates that RNA catalyzes protein synthesis, it seemed desirable to determine whether aminouracil mustard also suppressed the incorporation of uracil into the nuclear RNA, since it is conceivable that nuclear RNA might be a site of biosynthesis of the nuclear proteins. Several investigators have suggested that mustards might act directly on nucleic acids,[4, 5] and thereby interfere with cell metabolism and protein synthesis.[6] However, *in vivo* evidence for this possibility is still lacking.[7]

In the present studies, aminouracil mustard (4 mg/kg) was injected intraperitoneally into rats bearing the Walker 256 carcinosarcoma. At various intervals following the administration of the drug, uracil-2-^{14}C was injected intraperitoneally, and 75 min later the various intracellular fractions were isolated from tumor, spleen and liver from which RNA was extracted. These studies indicate that the aminouracil

* Much of the work described in this manuscript was carried out at the University of Illinois College of Medicine, to which we are indebted for the facilities and the opportunity to carry forward these studies.

mustard inhibits the incorporation of uracil-2-^{14}C into the nuclear RNA of the Walker 256 carcinosarcoma and the spleen.

MATERIALS AND METHODS

The animals used in these experiments were male rats, obtained from the Holtzman Rat Company (Madison, Wisconsin), weighing 200–250 g and fed *ad libitum* on Purina laboratory chow. The Walker 256 carcinosarcoma was transplanted from 7 to 10 days prior to the experiment. The uracil-2-^{14}C used in these experiments had a specific activity of 3·2 mc/m-mole (Volk Company, Chicago).

In each experiment 20 μc of the tracer were injected intraperitoneally into the tumor-bearing rat. The rat was anesthetized 75 min later by the intraperitoneal injection of 50 mg of sodium pentobarbital per kg and exsanguinated by means of heart puncture. The tissues were rapidly excised and placed in beakers containing ice-cold saline, and these were immediately transferred to the cold room (4 °C) where they were homogenized in istonic sucrose. The homogenates were subjected to differential centrifugation for the separation of the various intracellular fractions.[1] The nuclei of the tumor and the liver were isolated as recently described.[8]

For the isolation of the RNA a slightly modified phenol extraction procedure was used, based on the methods of Kirby[9], Goldthwait[10], and Hall and Doty[11]. The nuclear and cytoplasmic fractions were dialysed overnight against distilled water at 4 °C, while the other fractions were stored at −15 °C during this time. The dialysed fractions were then made 0·2 M with respect to potassium phosphate buffer, pH 6·8. The other fractions were taken up in 6 ml of 0·2 M phosphate buffer, pH 6·8. The fractions were then shaken for 30 min at room temperature with an equal volume of phenol saturated with water and centrifuged at 600 **g** for 30 min. The aqueous layer was siphoned off, and the entire procedure was repeated once. The two combined aqueous extracts were then shaken twice with ether, and chilled in the cold room at 4 °C. All fractions were made 0·2 N with respect to HCl and centrifuged after 1-hr incubation in the cold room at 4 °C at 600 **g** for 30 min. The white precipitates were washed once with 95 per cent ethanol and then were dissolved in 0·03 N ammonium hydroxide. The concentration of RNA was determined by the orcinol method,[12] while the radioactivity was determined by plating samples at infinite thinness. Each point on the graphs is the average of three experiments and in each experiment one rat was used. The specific activity of the mitochondrial RNA was essentially the same as that of the microsomal RNA and hence these data were omitted from the graphs. The 5-bis(2-chloroethyl) aminouracil was generously supplied by the Upjohn Company through the courtesy of Dr. H. G. Petering. The drug was injected intraperitoneally in single doses of 4 mg/kg in a solution containing 4 mg of the drug per ml of dimethylformamide.*

RESULTS

Studies on untreated animals

To obtain baseline values for comparison of results obtained in treated animals, the content of RNA was determined in the intracellular fractions of tissues of untreated animals (Fig. 1). The tumor and spleen appear to be very similar with respect to RNA distribution. The nuclear fraction of the liver contained one-fourth the quantity of

* Dimethylformamide, if administered in the same quantities, but without aminouracil mustard, did not inhibit the incorporation of uracil-2-^{14}C into the RNA of the various intracellular fractions of tumor, liver or spleen.

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RNA found in the tumor nuclei, while the microsomal fraction of the liver was richer in RNA than was that of the tumor. In the tissues studied, the microsomal fraction contained as much RNA as the remainder of the cell.

Kinetics of incorporation of uracil-2-^{14}C into RNA

The kinetics of the incorporation of radioactive uracil into RNA of the various intracellular fractions of the tumor, liver and spleen of the Walker tumor-bearing rat are shown in Figs. 2, 3 and 4, respectively. These data indicate a sharp initial rise in

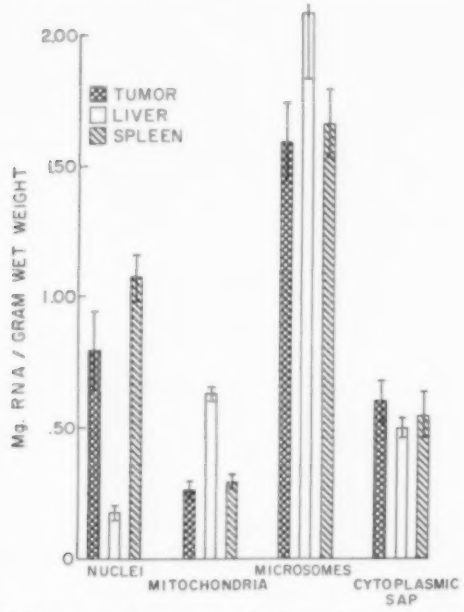

FIG. 1. Distribution of RNA in the various cellular fractions of tumor, liver and spleen in mg of RNA per g wet weight of tissue. The isolation of the RNA from the various cellular fractions was carried out according to the procedure described in the section on Materials and Methods. The results were obtained from nine experiments; the standard errors are indicated.

specific activity of nuclear RNA which tends to level off, or even decrease in the liver, after 1 hr.[13] In contrast, the specific activity of the RNA in the cytoplasmic fractions rises more slowly. In the tumor there was a lag period in the labeling of the RNA in the cytoplasmic fractions. The specific activity of the nuclear RNA of the tumor was the highest of the tissues studied at 180 min after injection of the tracer. The specific activity of the nuclear RNA of the liver reached a maximum at 75 min. As indicated in Fig. 5, the utilization of uracil is highest in the nuclear RNA of the tumor, as compared to the other tissues studied.[14, 15] At 75 min the RNA of the nuclear fractions of tumor, liver and spleen contained, respectively, 43, 57 and 33 per cent of the total counts per min per g of wet weight of tissue.

Kinetics of inhibition of labeling of RNA by aminouracil mustard

The kinetics of the inhibitory effects of the aminouracil mustard, in a dose of 4 mg/kg, on incorporation of uracil-2-^{14}C into the RNA of the Walker tumor, liver

and spleen, are presented in Figs. 6, 7 and 8, respectively. Fig. 6 indicates that there is a progressive suppression of the systems which incorporate the uracil into the nuclear RNA of the tumor. At 24 hr there was 50 per cent inhibition of labeling of nuclear RNA of the tumor. At this time, the labeling of the RNA of the cytoplasmic fractions was even more markedly inhibited than was the labeling of nuclear RNA. In the

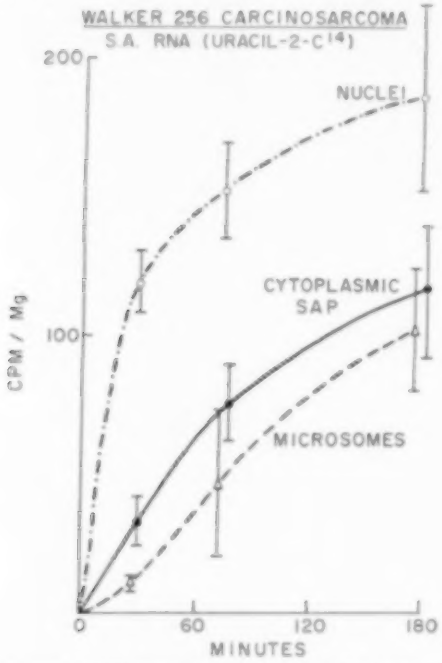

FIG. 2. Kinetics of the incorporation of uracil into the RNA of the various intracellular fractions of the Walker 256 carcinosarcoma. 20 μc of uracil-2-^{14}C were injected intraperitoneally. At the designated time, the tissues were excised from the anesthetized, exsanguinated animal and carried through the procedure indicated in the section on Materials and Methods. The standard errors are indicated.

spleen,* a progressive suppression of the labeling of the RNA in all fractions occurred until no radioactivity could be detected in any fraction after 24 hr. The rate of suppression of incorporation of uracil-2-^{14}C into the RNA of the spleen was greater than that of the tumor, and occurred at an earlier time. In the liver, the specific activity of the nuclear RNA increased to more than twice the control value at 12 hr after administration of the drug. The specific activity of the RNA in the cytoplasmic fractions rose slightly at this time point. None of the fractions studied showed a consistent change in the concentration of RNA as a result of the administration of aminouracil mustard.

DISCUSSION

Previously reported data concerning the inhibition by the aminouracil mustard of amino acid incorporation into the nuclear proteins of the Walker tumor and other

* As already reported,[3] the spleen of the animals shrink markedly in size after administration of aminouracil mustard. For this reason, the points at 12 hr after administration of the drug represent only two determinations, each of which was carried out on two to three pooled spleens in order to obtain a sufficient amount of tissue.

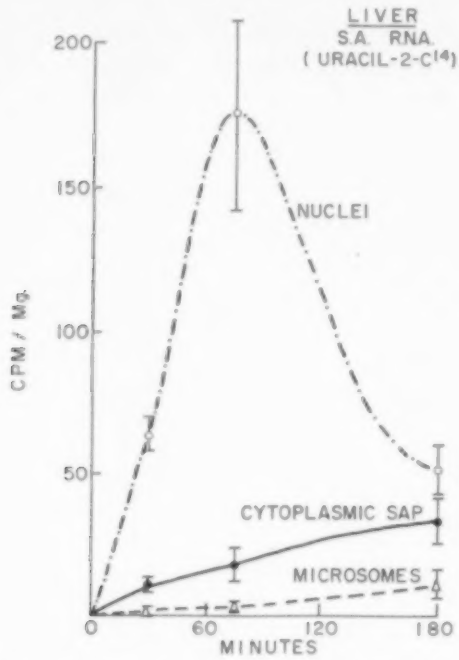

FIG. 3. Kinetics of the incorporation of uracil into the RNA of the various intracellular fractions of the liver. 20 μc of uracil-2-^{14}C were injected intraperitoneally. At the designated time, the tissues were excised from the anesthetized, exsanguinated animal and carried through the procedure indicated in the section on Materials and Methods. The standard errors are indicated.

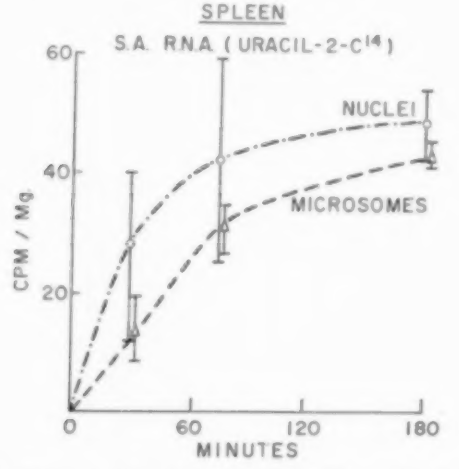

FIG. 4. Kinetics of the incorporation of uracil into the RNA of the nuclear and microsomal fractions of the spleen. 20 μc of uracil-2-^{14}C were injected intraperitoneally. At the designated time, the tissues were excised from the anesthetized, exsanguinated animal and carried through the procedure indicated in the section on Materials and Methods. The standard errors are indicated.

rat tissues, *in vivo*, suggested that the effects of the compound might occur selectively in the nucleus and particularly at sites involved in synthesis of acid-insoluble nuclear proteins.[3] Evidence for some intracellular specificity of action of the drug emerged from the finding that labeling of cytoplasmic proteins of tumor, liver and spleen was

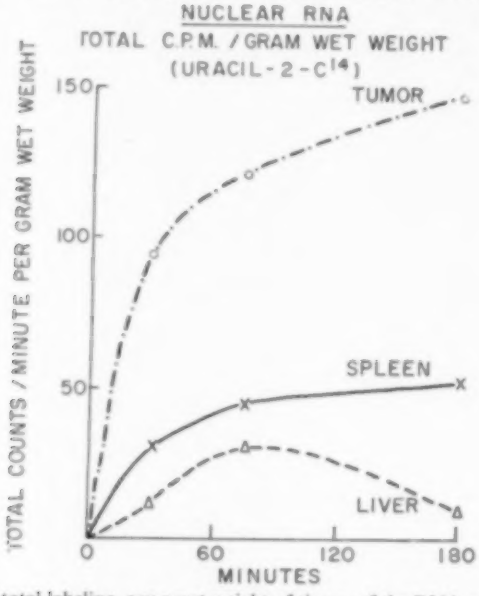

FIG. 5. Kinetics of the total labeling, per g wet weight of tissue, of the RNA of the nuclear fractions. The points were obtained by multiplying the average nuclear RNA content (Fig. 1) by the various specific activities.

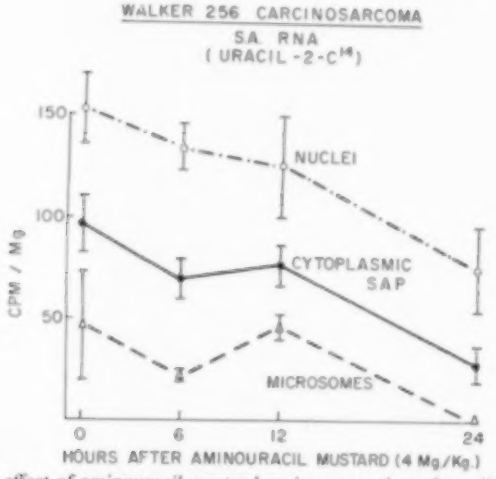

FIG. 6. Kinetics of the effect of aminouracil mustard on incorporation of uracil into the RNA of the various intracellular fractions of the Walker 256 carcinosarcoma; 75 min prior to the designated time, 20 μc of uracil-2-^{14}C were injected intraperitoneally. At the designated time, the tissues were excised from the anesthetized, exsanguinated animal and treated as indicated in the section on Materials and Methods. The standard errors are indicated.

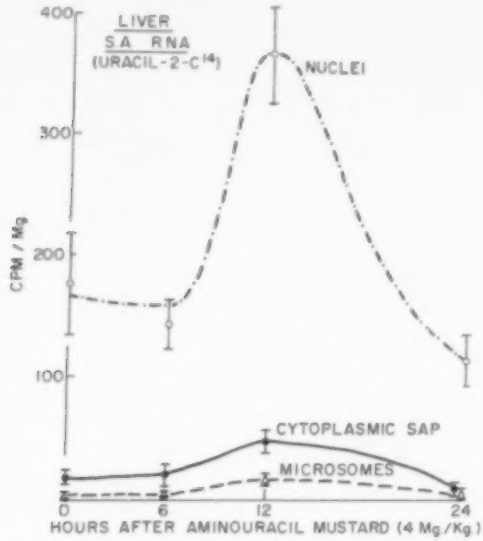

FIG. 7. Kinetics of the effect of aminouracil mustard on incorporation of uracil into the RNA of the various intracellular fractions of the liver; 75 min prior to the designated time, 20 μc of uracil-2-^{14}C were injected intraperitoneally. At the designated time, the tissues were excised from the anesthetized, exsanguinated animal and carried through the procedure indicated in the section on Materials and Methods. The standard errors are indicated.

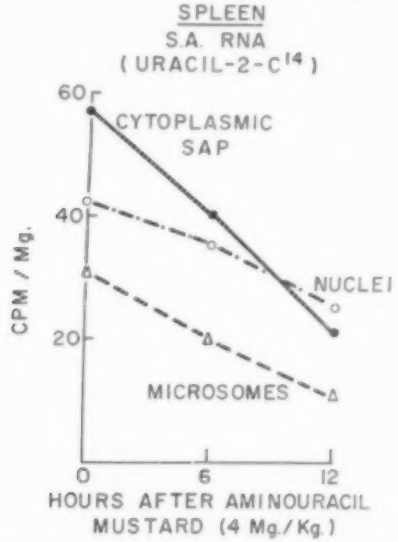

FIG. 8. Kinetics of the effect of aminouracil mustard on incorporation of uracil into the RNA of the various intracellular fractions of the spleen; 75 min prior to the designated time, 20 μc of uracil-2-^{14}C were injected intraperitoneally. At the designated time, the tissues were excised from the anesthetized, exsanguinated animal and carried through the procedure indicated in the section on Materials and Methods. The values and standard errors of these results at 0, 6 and 12 hr, respectively: nuclei, 42 $\pm$ 16·8, 25 $\pm$ 7, 25 $\pm$ 17; microsomes, 13 $\pm$ 5·5, 12 $\pm$ 9, 0; cytoplasmic sap, 40 $\pm$ 11·1, 21 $\pm$ 7, 0.

not inhibited, while the labeling of the nuclear proteins, particularly of the acid-insoluble nuclear proteins, was markedly decreased in tumor and spleen.

The present report indicates that the suppression of incorporation of uracil into the nuclear RNA of the Walker tumor by the aminouracil mustard was less marked than was the inhibition of incorporation of amino acids into the acid-insoluble nuclear proteins. It remains to be determined whether this suppression of uracil incorporation into RNA reflects a general suppression of incorporation of precursors into RNA or a more specific effect on uracil incorporation into RNA. However, the suppression of labeling of the nuclear RNA preceded the drop in labeling of the acid-soluble nuclear proteins. In the spleen, the incorporation of labeled arginine into the nuclear proteins was also inhibited to a greater extent in the first 6 hr after administration of the drug than was the incorporation of labeled uracil into the nuclear RNA. In contrast to the results obtained with L-arginine-U-^{14}C, the incorporation of uracil into the RNA of the *cytoplasmic* fractions was markedly suppressed. This result is consistent with the current concept that most if not all of the cytoplasmic RNA originates in the nucleus.[16, 17]

The intracellular selectivity of action of aminouracil mustard with regard to incorporation of labeled amino acids into the nuclear proteins of the Walker tumor, was not paralleled by a similar selectivity for the incorporation of uracil into the RNA of the various cellular fractions. As indicated previously, this result would be expected if the nucleus is the site of synthesis of most or all of the cellular RNA. This concept would imply that the primary site of action of the aminouracil mustard on biosynthesis of RNA is also in the nucleus. The results obtained do not rule out a role for nuclear RNA in the biosynthesis of acid-insoluble nuclear proteins, since a number of alternative explanations exist for these results. For example, the drug may attack existing molecules of nuclear RNA and destroy their catalytic activity; alternatively, the mustard may induce formation of abnormal RNA or suppress the formation of special RNA necessary for biosynthesis of the acid-insoluble nuclear proteins. It is evident that more knowledge needs to be obtained regarding nuclear RNA and its functions. With the aid of chromatographic methods, now becoming available, progress in this field may be accelerated.[10]

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A STUDY ON THE TOXICITY AND THE BIOCHEMICAL EFFECTS OF MONOFLUOROMALONATE

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Abstract—The toxicity of monofluoromalonate (FM) to guinea pigs, rats and mice has been examined. The symptoms of poisoning observed are similar to those obtained with fluoroacetate (FAc), but the LD_{50} of FM is found to be higher by an order of magnitude. The action of FM is accompanied by an accumulation of citrate in various tissues, the effect being most pronounced in the kidney.

It is shown that, under the experimental conditions employed, spontaneous decarboxylation of FM to FAc is negligible. However, evidence is presented that, with guinea pig kidney particles, the conversion of FM to FAc does take place. The experimental results substantiate the assumption that the metabolic effects of FM are due, at least partly, to FAc produced as an intermediary by enzymatic decarboxylation.

The inhibitory effect of FM on succinic dehydrogenase and oxaloacetic decarboxylase proves to be markedly smaller than that of malonate.

INTRODUCTION

IN THIS study a comparison is made between the metabolic pathway of malonate and that of monofluoromalonate (FM). The latter compound has been synthesized recently by Bergmann *et al.*[1] in the form of both its mono-potassium salt (KHFM), and its diethyl ester (DEFM).

The enzymatic decarboxylation of malonic acid was studied in *Pseudomonas fluorescens* by Hayaishi[2] and in rat tissues by Nakada *et al.*[3] Later work by Wakil[4] and by Formica and Brady[5], identified malonyl-CoA as an intermediate in fatty acid synthesis and established its formation by the enzymatic carboxylation of acetyl-CoA. According to these authors, the mechanism of malonate degradation may be summarized as follows:

$$\text{malonate} \longrightarrow \text{malonyl-CoA} \longrightarrow \text{acetyl-CoA} + CO_2$$

Assuming an analogous mechanism for the degradation of FM, the corresponding intermediate will be fluoroacetyl-CoA, i.e. the effect of FM is expected to be similar to that of fluoroacetate (FAc). The experiments carried out for testing this assumption are based on the investigations of Peters and his colleagues[6, 7] who have shown that, both *in vivo* and *in vitro*, FAc can be metabolized to fluorocitrate which causes accumulation of citrate.

Furthermore, since malonate is a well-known inhibitor of both succinic dehydrogenase and oxaloacetic decarboxylase, the effect of FM on these two systems was also investigated.

MATERIALS AND METHODS

KHFM and DEFM were supplied by Dr. E. D. Bergmann and Dr. S. Cohen.

For the *in vivo* experiments, KHFM was neutralized to pH 7·0 with KOH and diluted with saline to the appropriate concentration; DEFM was dissolved in propylene glycol to which saline was added in the ratio of 6 : 4. The injections were administered subcutaneously.

For the *in vitro* experiments, kidney (guinea pigs) and brain (pigeon) particles were prepared according to O'Brien and Peters[8]. Each flask contained: 1·0 ml enzyme preparation; 125 μmoles phosphate buffer pH 7·2; 1·2 μmoles disodium adenosine triphosphate (ATP); 4·0 μmoles $MgCl_2 \cdot 6H_2O$. Substrate for kidney: 22 μmoles fumarate. Substrate for brain: 32 μmoles sodium pyruvate plus 4·4 μmoles sodium fumarate. Various amounts of either KHFM (neutralized to pH 7·2) or FAc were added; in another set of experiments 1·2 ml of "kidney supernatant" was added instead. In all cases the volume was made up to 3·5 ml with 0·9 per cent KCl solution and incubation carried out at 38 °C. Gas phase: air.

Preparation of "kidney supernatant": Kidney particles were incubated in the usual way for 30 min with various amounts of either KHFM or FAc. After incubation the contents of the flask was heated in boiling water for 5 min, cooled, and centrifuged. The clear supernatant was used.

Succinic dehydrogenase was assayed either manometrically or colorimetrically using the tetrazolium method.[9] A 10 per cent (w/v) of rat kidney homogenate in 0·2 M phosphate buffer (pH 7·4) served as enzyme preparation. Oxaloacetic decarboxylase was assayed according to Ochoa[10]. Citrate was estimated by the method of Beutler and Yeh[11]. Glucose was determined according to the method of Nelson[12].

In all the manometric experiments the conventional Warburg techniques[13] were employed.

RESULTS

Spontaneous decarboxylation of FM

Since malonate forms acetate by spontaneous decarboxylation, it was of importance to ensure that the FM used did not contain any FAc. Hence, separate examinations were carried out with the following results:

(1) Chromatographic analysis[15] of KHFM showed that only traces of FAc were present (less than 0·1 per cent).

(2) Incubation of KHFM for 3 hr (in the usual medium without substrate and enzyme), at concentrations up to 1000 μmoles/flask did not cause any measurable liberation of CO_2.

(3) Heating of KHFM solution at 100 °C for 30 min did not change its toxicity to rats.

These three results prove that, in all the experiments performed, the amount of FAc formed by spontaneous decarboxylation is negligible.

Effects in vivo

KHFM and DEFM were injected subcutaneously to guinea pigs, mice, and rats in order to determine the LD_{50}. Guinea pigs were found to be far more sensitive than either mice or rats. Mice showed a higher sensitivity to DEFM than to KHFM[14] whereas in rats no difference between the two compounds was observed. The results

of the LD_{50} determinations are summarized in Table 1, which also includes the corresponding values for FAc.

Comparison of the first and third columns in Table 1 shows that the toxicity of KHFM—the only FM compound used in the following experiments—is 6–12 times lower than that of FAc.

TABLE 1. TOXICITY OF KHFM AND DEFM TO MICE, RATS, AND GUINEA PIGS

Animal*	LD_{50} (mg/kg)		
	KHFM	DEFM	NaFAc
Mouse	80	15	15
Rat	60	70	5
Guinea pig	2	—	0·25

* Six animals were used in each group.

The following symptoms of poisoning were observed in rats and mice injected with either DEFM or KHFM: after 15–20 min the animals became lethargic with their hair standing on end. About 2 hr later their rectal temperature decreased and their heart beats slowed down. The animals sprawled on their hind legs, stopped eating and drinking, and showed periodic convulsions.

No ketone bodies were detected in the urine of the injected animals whereas a rise in their blood glucose occurred, up to 2–2·5 times the normal value. Death was associated with a considerable accumulation of citrate in the different mouse tissues examined (kidney, heart, brain, liver and diaphragm), the increase being particularly marked in the kidney. However, when the injection of KHFM was immediately

TABLE 2. EFFECT OF KHFM AND MALONATE UPON CITRATE LEVELS IN DIFFERENT MOUSE TISSUES

Tissue	Citric acid (μg/g wet tissue)			
	Normal	KHFM	Malonate	KHFM + malonate
Kidney	16 ± 2	1150 ± 184	20 ± 3	363 ± 115
Heart	40 ± 4	302 ± 55	70 ± 5	186 ± 24
Brain	20 ± 2	207 ± 80	20 ± 2	175 ± 37
Liver	5 ± 1	65 ± 10	10 ± 2	40 ± 7
Diaphragm	70 ± 5	300 ± 40	225 ± 40	270 ± 106

Groups of five animals were used. Amount of KHFM injected: 60 mg/kg; of sodium malonate: 1·5 g/kg. Citrate tests carried out 1½ hr after injection. Figures are means ± standard deviations.

followed by an injection of malonate, the increase in the citrate level of heart and kidney was reduced, the kidney again being the most affected organ (Table 2). Similar results were obtained with rat tissues.

Effects in vitro

1. *Kidney particles.* Because of the higher sensitivity of guinea pigs to KHFM, the kidneys of these animals were chosen for the enzymic measurements.

KHFM, in the presence of fumarate, caused an accumulation of citrate and an inhibition of respiration (Fig. 1). However, the two effects differed with respect to their dependence upon the concentration of KHFM: While the inhibition of oxygen uptake increased monotonically with concentration in the whole range examined, the citrate content showed a rather flat maximum at a concentration of 4–5 μmoles/flask.

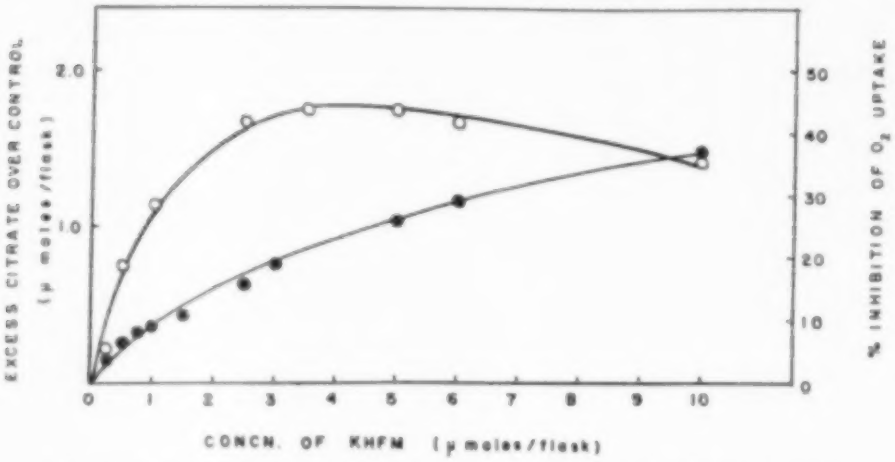

FIG. 1. Effect of KHFM on citrate accumulation (○) and respiration (●) of kidney particles.

The effect of KHFM was found to be counteracted by malonate, e.g. at a KHFM concentration of 4 μmoles/flask, addition of 2 μmoles malonate decreased the citrate content by 37 per cent and increased the oxygen uptake by 56 per cent.

It is of interest to mention in this connexion that addition of acetate instead of malonate produced variable results, e.g. 2 μmoles of acetate decreased citrate content by about 10 per cent while 5 or 10 μmoles acetate caused an increase of about the same percentage (concentration of KHFM, 4 μmoles/flask).

2. *Pigeon brain particles.* The results for the action of KHFM on pigeon brain particles with respect to respiration and citrate accumulation are summarized in Table 3(a). Values obtained with FAc have been included for comparison.

TABLE 3(a). EFFECTS OF KHFM AND FAc ON PIGEON BRAIN PARTICLES

Inhibitor	Concentration (μmoles/flask)	O_2 uptake (μmoles/30 min)	Citrate content (μmoles)
KHFM	0	3·1	0·33 ± 0·04
KHFM	1·0	3·0	0·41 ± 0·06
KHFM	3·0	2·9	0·46 ± 0·06
KHFM	5·0	2·7	0·51 ± 0·07
KHFM	10·0	—	0·50 ± 0·12
FAc	0·5	—	0·32 ± 0·04
FAc	1·0	—	0·37 ± 0·07
FAc	2·0	—	0·41 ± 0·07

Time of incubation 1 hr. Figures are means ± standard deviations.

It may be seen from the table that the inhibitory effect of KHFM on respiration was slight. The citrate content increased significantly (on the 5 per cent level) only at a concentration of 5 μmoles/flask; at lower and higher concentrations of KHFM the increase was not significant. Similarly, in agreement with the results of Peters and Wakelin[16], FAc did not cause any accumulation of citrate. On the other hand, these authors observed that fluorocitrate, when substituted for FAc, induced marked citrate accumulation in pigeon brain particles. Therefore, they attributed the toxic effects of FAc in guinea pig kidney particles to the fluorocitrate metabolically formed from FAc.

Assuming that FM incubated with kidney particles is degraded to FAc and then metabolized to fluorocitrate, the latter compound should be present in the "kidney supernatant" obtained with KHFM. The validity of this assumption was examined by incubating the "kidney supernatant" with pigeon brain particles. If any fluorocitrate is formed in the kidney supernatant, a notable accumulation of citrate[16] should occur. The results are presented in Table 3(b).

TABLE 3(b). EFFECTS OF "KIDNEY SUPERNATANT" ON PIGEON BRAIN PARTICLES

Inhibitor used for preparation of K.S.*	Conc. of inhibitor (μmoles/flask)	O_2 uptake (μmoles/30 min)	†Citrate content (μmoles)
KHFM	0‡	5·1	0·54 ± 0·06
KHFM	0·5		0·58 ± 0·05
KHFM	1·0	4·9	0·92 ± 0·10
KHFM	2·0	4·5	1·01 ± 0·11
KHFM	3·0		1·06 ± 0·09
KHFM	5·0		1·35 ± 0·07
FAc	0·5	4·6	0·84
FAc	1·0	4·4	1·51
FAc	2·0	3·6	1·65

Time of incubation 1 hr.
* K.S. kidney supernatant.
† Original citrate content of added K.S. has been subtracted. Figures are means ± standard deviations.
‡ Compare first row of Table 3(a) for control without K.S.

In agreement with the assumption, the table shows a remarkable increase in citrate content caused by the action of "kidney supernatant". The increase rose monotonically with the concentration of KHFM used for the preparation of "kidney supernatant". Values obtained with FAc are included for comparison.

3. *Production of* CO_2 *from KHFM.* According to the assumed mechanism for the degradation of FM, CO_2 should be evolved concurrently. In order to determine whether CO_2 was in fact liberated and to measure its amount per hour, the following experiments were repeatedly carried out in the Warburg apparatus: kidney particles in the usual medium without substrate were incubated with KHFM at concentrations of 0–100 μmoles/flask, the gas phase being nitrogen. The results, duly corrected for the liberation of CO_2 from the control, are summarized in Table 4.

It may be seen from the table that, in the range of concentrations usually employed in this study, the amount of CO_2 liberated increased with concentration of KHFM.

Further increase in KHFM concentration, even up to 100 μmoles/flask, caused only a very slight further increase in the amount of CO_2 evolved.

These results substantiate the assumption that the effects of KHFM described in this study are associated with its degradation to FAc, CO_2 being evolved.

TABLE 4. LIBERATION OF CO_2 FROM KHFM ACTING ON KIDNEY PARTICLES

Conc. of KHFM (μmoles/flask)	CO_2 liberated* (μmoles/hr)
0	0·1 ± 0·1
2	0·3 ± 0·1
10	1·0 ± 0·1
50	1·2 ± 0·2
100	1·5 ± 0·3

Temp. of incubation 38 °C.
Period of incubation 1 hr.
The results are means ± standard deviations.
* Excess over control.

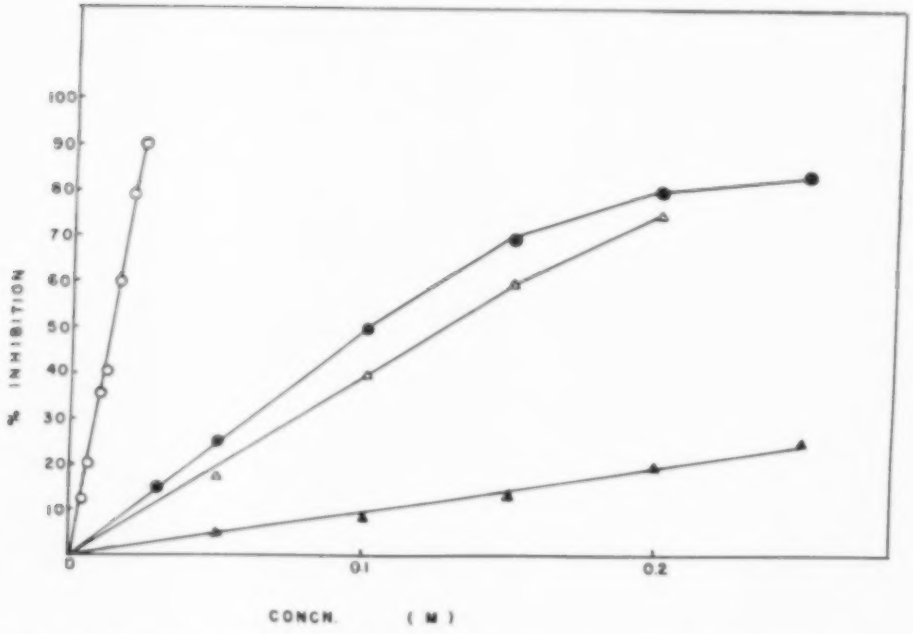

FIG. 2. Inhibitory effect of malonate (○), KHFM (●), methylmalonate (△) and dimethylmalonate (▲).

4. *Inhibitory effect of KHFM on succinic dehydrogenase and on oxaloacetic decarboxylase.* The results for the inhibitory effect on succinic dehydrogenase are summarized in Fig. 2. Parallel measurements for sodium malonate, sodium methylmalonate, and sodium dimethylmalonate are included for comparison. The values of

Fig. 2 were obtained by the colorimetric method.[9] The Warburg technique gave similar results. Fig. 2 shows that malonate was about ten times more effective than KHFM, the inhibiting action of methylmalonate and dimethylmalonate being even weaker than that of KHFM.

Experiments were carried out to compare the effect of malonate to that of KHFM on oxaloacetic decarboxylase at an identical concentration (10 μmoles/flask). Malonate proved to be almost twice as effective as KHFM (percentage inhibition by malonate, 35, by KHFM, 20).

DISCUSSION AND CONCLUSIONS

The experimental results conform with the assumption that the degradation of FM is analogous to that of malonate;[2–5] i.e. there appears to be no doubt that FAc (or fluoroacetyl-CoA) is an intermediate metabolite. However, it may be supposed that the conversion of FM to FAc is not complete and that possibly other intermediaries formed also play an important part. This supposition is based on the fact that, although the toxic action of FM (*in vivo* and *in vitro*) resembles that of FAc, the former requires higher concentrations than the latter for producing similar effects. Moreover, in the range of FM concentrations usually employed in this study (0–10 μmoles/flask), only about 10 per cent of the equivalent amount of CO_2 is liberated (Table 4).

It is to be pointed out, though, that no decisive proof could be given for the presence of fluoroacetyl-CoA: No fluoroacetyl hydroxamate could be detected by the method of Lipmann and Tuttle[17] when hydroxylamine trapping experiments[18] were carried out.

Malonate has proven to be a strong suppressor, *in vivo* and *in vitro*, of the effect of KHFM with respect to citrate accumulation in the kidney. It is not clear, to what degree the malonate influences the metabolic decarboxylation of FM to FAc. The action of "kidney supernatant", obtained by incubation of KHFM + malonate, on pigeon brain particles has not been examined. It is probable that the effect of the added malonate is due to the blocking of fluorocitrate formation[16] and, therefore, citrate accumulation in pigeon brain particles should also decrease significantly. Further investigation of these problems is indicated.

The comparison of the inhibitory influence on succinic dehydrogenase of malonate, KHFM, methylmalonate, and dimethylmalonate (Fig. 2) is of particular interest. Since the activity of KHFM lies between that of malonate and methylmalonate, one may assume that the size of the ligands of the central carbon atom is the determining factor: the larger their size, the lower the activity. It is an accepted fact that often the similarity in size between hydrogen and fluorine determines the biological activity of fluorinated compounds.

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NEUROTOXIC AND BIOCHEMICAL PROPERTIES OF SOME TRIARYL PHOSPHATES

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Abstract—A number of tri(alkyl-substituted phenyl) phosphates have been examined for neurotoxicity to chickens, and for inhibition *in vitro* and *in vivo* of pseudocholinesterase and esterase. No correlation was found between chemical structure and neurotoxicity. Inhibition *in vivo* of chicken plasma cholinesterase was caused by many of these compounds. It was possible to predict the degree of inhibition produced by the unsymmetrical esters from the results obtained with symmetrical tri-esters. Tri-4-methyl- and tri-4-ethylphenyl-phosphates were potent inhibitors *in vitro* of liver and intestinal phenyl butyrate esterase while the corresponding tri-2-methyl and tri-2-ethyl analogues were not. *In vivo* all four compounds caused inhibition of this esterase activity.

INTRODUCTION

OVER the last 60 years many cases of poisoning involving damage to the nervous system have occurred in men and women who have ingested tricresylphosphates, under a variety of circumstances. Smith *et al.*[1] first showed that tri-2-methylphenylphosphate (tri-*o*-cresylphosphate, tri-*o*-tolylphosphate, TOCP) was the toxic agent in these mixtures and demonstrated the value of the hen as a sensitive species for testing the neurotoxicity of these materials.

Recently, Hine *et al.*[2] and Henschler[3, 4] and Henschler and Bayer[5] have examined more of the analogues of TOCP including the tri-dimethylphenylphosphates (tri-xylenylphosphates). Bondy *et al.*[6], in a quest for a non-toxic tri-aryl phosphate plasticizer that could be produced commercially, examined a number of compounds that were present as impurities in commercial tolyl and xylenyl phosphate mixtures.

The work reported in this paper was carried out largely on the compounds prepared and tested by these workers. The observations on neurotoxicity have been supplemented by some studies on the biochemical activity of these compounds. The implications and the findings will be discussed but no attempt will be made in this paper to review the whole problem of the neurotoxicity of the organophosphorus compounds (see Barnes and Denz[7], Davies *et al.*[8]).

MATERIALS AND METHODS

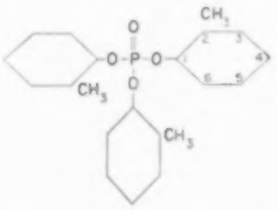
Hens

Young chicks are insensitive to the neurotoxic action of organo-phosphorus compounds (Barnes and Denz[7], Bondy *et al.*[6]), so that hens at least 4 months old and usually 6–12 months of age were used. Rhode Island Red, Rhode Island Red × Light Sussex and White Leghorns were obtained from commercial breeders. In addition F_1

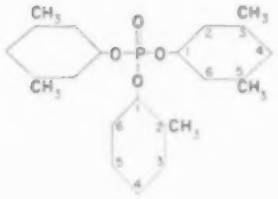
in-cross hens from two pure lines bred at the A.R.C. Station, Reeseheath, were used. These birds, when the same age, showed a smaller range of individual variation in their plasma cholinesterase levels than other birds even from a closed flock (Table 1). The pure line birds also showed a gradual fall of blood cholinesterase activity with age.

TABLE 1. THE PLASMA CHOLINESTERASE ACTIVITY (μL CO_2/MIN PER ML PLASMA AT 37 °C ACH SUBSTRATE) IN HENS FROM DIFFERENT SOURCES AND AT DIFFERENT AGES

Source	Age	No.	Plasma cholinesterase	Coefficient of variation (%)
1. Commercial birds	Adult	62	16·5 ± 4·81	29·2
2. Closed flock	Adult	6	18·3 ± 4·47	24·4
3. F_1 incross hens (A.R.C. Station, Reeseheath)	126–136 days	13	21·3 ± 2·42	11·3
	143–154 days	13	20·3 ± 1·53	7·5
	156–161 days	10	18·4 ± 2·18	11·8
	180 days	12	17·5 ± 1·59	9·1
	200–210 days	12	13·9 ± 1·45	10·4
	250–280 days	10	16·1 ± 1·85	11·5
Total	4–8 months	70	18·02 ± 3·12	17·2



Tri 2-methylphenylphosphate



Mono 2-methylphenyl, di 3:5-dimethylphenylphosphate

FIG. 1. Examples of structural formulae of a symmetrical and unsymmetrical tri(alkyl substituted phenyl) phosphate.

Compounds

The compounds studied in this paper are either symmetrical tri(alkyl-substituted phenyl)phosphates or phosphate esters containing two identical phenyl residues (B) and one other (A). The elemental analysis and physical characteristics of these compounds have been reported by Bondy *et al.*[6] Since no methods are available for the determination of the molecules as a whole the possibility of impurities must be deduced

from the methods of synthesis. The symmetrical tri(substituted phenyl)phosphates were prepared from the highly purified phenols by reaction with $POCl_3$ followed by isolation and fractionation by distillation or crystallization. For the preparation of the mixed esters (mono-A-, di-B-phosphates) the general method was to react phenol-B with $POCl_3$, fractionate the product to obtain di-B-phosphoryl chloride and then react this with the Na salt of phenol-A. The product was then finally purified, usually by distillation. The purity of the final product depends upon the degree of fractionation obtained at the various stages. The following example (Bondy, unpublished) gives the boiling points of the various intermediates which might be formed by reaction of 2-ethylphenol with $POCl_3$. It is clear that $POCl_3$ will not be present after

TABLE 2. BOILING POINTS OF POSSIBLE PRODUCTS OF THE REACTION OF 2-ETHYLPHENOL WITH $POCl_3$

Compound	B.P. (°C)	Pressure (mm Hg)
$POCl_3$	107·2	760
Mono-2-ethylphenyl phosphoryldichloride	approx. 80	0·5
Di-2-ethylphenyl phosphorylchloride	approx. 165	0·5
Tri-2-ethylphenylphosphate	221–223	1·0

fractionation of di-B-phosphoryl chloride and therefore the final product will not contain tri-A-phosphate. The boiling points of the remaining intermediates are sufficiently far apart for fractionation to separate almost completely di-B-phosphoryl chloride from mono-B-phosphoryldichloride and tri-B-phosphate but traces might remain. The most likely impurities by this route of preparation of mono-A-, di-B-phosphate are therefore mono-B-, di-A-phosphate and tri-B-phosphate. When prepared by the alternative route through mono-A-phosphoryl dichloride (only mono-2-methylphenyl-, di-3:5-dimethylphenylphosphate and mono-2-ethylphenyl-, di-3:5-dimethylphenylphosphate were prepared by this route), a possible impurity is mono-B-, di-A-phosphate. Such assumptions are only valid if no rearrangement of the groups takes place during the preparation and final fractionation.

Many specimens of tri-2-methylphenylphosphate (TOCP) inhibit plasma cholinesterase *in vitro*.[9–11] This inhibition is not due to the parent compound but to an impurity.[12] All the compounds used in this paper have therefore been tested for inhibitory power *in vitro* against chicken plasma cholinesterase. Plasma (0·5 ml) was incubated for 15 min at 37 °C with 9×10^{-5} M compound (this concentration in water gives a faintly opalescent solution and is therefore saturated). The range of the control cholinesterase was 15·6–16·4 μl CO_2/min per ml plasma (5 per cent range). With two exceptions all the compounds gave less than 5 per cent inhibition. Tri-3-methylphenylphosphate gave 5 per cent and tri-2-methylphenylphosphate 6 per cent inhibition. It may be concluded that these compounds are substantially free from impurities inhibitory to chicken plasma cholinesterase.

Methods

All the compounds studied are virtually insoluble in water. After some trials with intraperitoneal injections it was decided to give them all by mouth. Solid esters were

dissolved either in arachis oil or, where this was not possible, in a mixture of tri-4-methylphenylphosphate and tri-3-methylphenylphosphate (20:80 or 40:60). Evidence of absorption after dosing by mouth was obtained:

(a) By estimating the total ether-soluble P in dried faeces collected for 36 hr after dosing. Results with a range of compounds indicated that at least 50–80 per cent of the dose was absorbed, or (less likely) broken down in the gut. The exact nature of the excreted P was not determined.

(b) By estimating the blood cholinesterase activity before and after a single dose. The fall which was seen with the majority of compounds studied was evidence of absorption.

Tri-2-methylphenylphosphate was shown to be equally effective in producing ataxia whether given as solutions in arachis oil or in the inert aryl phosphate solvent.

Determination of enzymes

The cholinesterase activity of heparinized plasma (0·5 ml) was determined by a manometric method using acetylcholine (0·0138 M) as substrate and in a medium (total volume 4 ml) containing NaCl (0·131 M), $MgCl_2$ (0·035 M) and $NaHCO_3$ (0·0312 M) and gassed with 5 per cent CO_2 in N_2 at 37 °C.[13, 14] Brain pseudo cholinesterase activity was calculated from the rates of hydrolysis of acetylcholine (0·0138 M) and *n*-butyryl choline (0·03 M). From the known ratios of the rates of hydrolysis of these substrates by true and pseudocholinesterase of chicken brain, the activity of each enzyme against either substrate may be calculated.[15, 16]

Esterase activity of chickens' liver and intestine was determined using phenylbutyrate (1·7 per cent, v/v) as substrate in the medium described above for cholinesterase determination but containing in addition 0·5 per cent (w/v) gelatin. Tissue was homogenized in the medium (without gelatin) to give a 10 per cent (wet w/v) homogenate. Esterase activities were determined on suitable dilution and were expressed as μl CO_2/min per mg protein. The protein content of the homogenates was determined by a modification[17] of the biuret method of Robinson and Hogden[18]. Protein was precipitated by adding the homogenate (1–2 ml), to approx. 10 ml alcohol–ether mixture (50:50 v/v). After precipitation and centrifugation the pellet was resuspended in alcohol–ether and recentrifuged. The pellet was finally dissolved in NaOH, the protein determined by the biuret method and the results expressed as mg of albumin.

For experiments *in vitro* the compounds were dissolved in dimethylformamide. Various dilutions (0·03 ml) were added to the enzyme (3 or 3·5 ml). The concentration of dimethylformamide during the incubation with inhibitor never exceeded 1 per cent (v/v). This concentration had no effect upon chicken plasma cholinesterase or liver and intestinal esterase. Since the inhibition of esterase is dependent upon the time of incubation, enzymes were always incubated at 37 °C for 15 min prior to the addition of substrate.

Plasma cholinesterase

Determinations were made before, 24 hr and 4 days after a single dose in the majority of instances. There was evidence of absorption within a few hours of a dose by mouth. In every case activity had returned to normal levels within 4 days of an oral dose. However, in four birds given a non-neurotoxic compound (tri-2-ethylphenylphosphate) by intraperitoneal injection, return of plasma cholinesterase activity

did not take place for many weeks, indicating that the compound must have been absorbed very slowly from this site.

RESULTS

1. *Neurotoxic action*

This was judged entirely by inspection of the birds and no pathological examination was carried out on animals used in these experiments. Birds were observed for at least 21 days after a single dose and evidence of ataxia sought. The earliest signs were usually some unsteadiness when standing after the birds had been made to walk. This might be difficult to judge at an early stage but in true poisoning the disability would

TABLE 3. THE NEUROTOXIC ACTION OF TRI(SUBSTITUTED)PHENYLPHOSPHATES GIVEN BY MOUTH TO ADULT HENS

Substituents	Dose (mg/kg)	Neurotoxicity
None	500	0/4
Tri-2-Me	200–250	4/6
	100	0/9
Mono-3-Me, Di-2-Me	250	2/2
	50	0/2
Mono-2-Me, Di-4-Me	250	2/2
	50	3/4
Mono-2-Me, Di-3:5-di-Me	1000	2/2
	500	0/2
Tri-3-Me	2000	0/2
Tri-4-Me	15 × 500	0/2
Tri-2-Et	1000	0/4
Mono-4-Me, Di-2-Et	500	2/2
Mono-2-Et, Di-4-Me	200	2/2
Mono-2-Et, Di-3:5-di-Me	1000	2/2
	500	1/2
Tri-3-Et	1000	0/1
Mono-4-Et, Di-3-Et	500	0/2
Tri-4-Et	1000	3/3
	200	1/1
Mono-4-Me, Di-4-Et	500	0/2
Mono-4-Et, Di-4-Me	5 × 500	0/2
Mono-4-Et, Di-3:5-di-Me	5 × 500	0/2
Tri-2-*n*-Pro	1000	0/2
Mono-4-Me, Di-2-*n*-Pro	4 × 500	0/2
Mono-2-*n*-Pro, Di-4-Et	1000	2/2
	200	1/2
Mono-2-*n*-Pro, Di-3:5-di-Me	1000	0/2
Tri-2-Cl	1000	0/2
Tri-2-Me-4-Et	500	0/2
Miscellaneous compounds		
Tetra-2-Me-phenylpyrophosphate	1000	0/2
Di-*n*-propoxy, Mono-2-Me-phenylphosphate	1000	0/2

invariably progress significantly within 2–3 days of its onset being first suspected. Ataxia never first appeared later than 14 days after a single dose.

In severe cases the bird became unable to stand within a few days of the onset of signs of poisoning. In milder cases ataxia would not progress to this stage but remain conspicuous for several weeks and then slowly improve. Weight loss was not consistently seen until the birds became very helpless. In Table 3 the compounds studied

have been listed together with the doses shown to be either toxic or non-toxic. In addition to these, the five symmetrical tri-dimethylphenylphosphate esters (2:3, 2:5, 2:6, 3:4, 3:5) were found to be non-toxic in doses of 500 mg/kg. As there seemed to be no overall correlation between chemical structure and neurotoxicity it seemed to be worthwhile looking for some correlation between biochemical properties and either chemical structure or neurotoxic activity.

2. *Cholinesterase inhibition and toxicity*

The hypothesis put forward by Earl and Thompson[11, 19] based on their study of tri-2-methylphenylphosphate (TOCP) was that the inhibition of pseudocholinesterase was probably related to the myelin damage seen in those birds poisoned with TOCP Davison[15] showed that for the alkyl phosphate esters as a whole no such correlation could be established since compounds that were not neurotoxic could depress brain pseudocholinesterase to the same degree and for as long as the neurotoxic compounds. We had found that the triaryl phosphate ester, tri-4-ethylphenylphosphate which was neurotoxic produced little depression of pseudocholinesterase whereas the non-neurotoxic ester tri-2:5-dimethylphenylphosphate inhibited pseudocholinesterase.

TABLE 4. A COMPARISON OF THE EFFECTS ON PLASMA AND BRAIN PSEUDO-ChE ACTIVITY AND THE NEUROTOXICITY OF THREE TRI(SUBSTITUTED)PHENYLPHOSPHATES GIVEN BY MOUTH TO TWO HENS

(Dose 300 mg/kg of solution in arachis oil.)

	Percentage normal activity				
Time after dosing	24 hr		14–21 days		Condition
Substituents	Blood	Brain	Blood	Brain	
Tri-2:5-dimethyl	15	55	115	70	Normal
	29	69	110	48	Normal
Tri-4-ethyl	92	88	117	86	Ataxic
	105	88	130	90	Ataxic
Tri-2-methyl	0	25	105	75	Ataxic
	0	23	140	57	Ataxic

The data with three compounds is given in Table 4. It is clear that there is no correlation among the triarylphosphates between the power to inhibit cholinesterase and the ability to produce neurotoxic damage.

3. *Cholinesterase inhibition and chemical structure*

Although tri-2-methylphenylphosphate is able to reduce blood and tissue cholinesterase activities to very low levels in the animal, it was shown earlier[12] that purified specimens have no such activity *in vitro*. All the compounds considered here are free of any anticholinesterase inhibitory activity *in vitro*. Many, but not all, produce cholinesterase inhibition in the whole animal. The data for symmetrical tri-arylphosphates are summarized in Table 5, and it has been possible to divide the compounds into three groups depending on the degree of inhibition produced by a comparable single oral dose. In every case this inhibition must have been produced by a metabolite and the change must involve one or more of the phenyl rings. No

suggestion about the nature of this change can be offered but the properties of the mixed esters show some interesting features. Where the symmetrical ester is active in the hen as an inhibitor of cholinesterase so also will any unsymmetrical ester show inhibitory activity provided it contains at least one aryl group of an active symmetrical tri-ester (Table 6).

TABLE 5. THE EFFECT OF SYMMETRICAL TRI(SUBSTITUTED)PHENYLPHOSPHATES UPON BLOOD CHOLINESTERASE OF HENS 24 HR AFTER A SINGLE ORAL DOSE

(Group A, compound producing <25 per cent inhibition; group B, compound producing 25–75 per cent inhibition; group C, compound producing >75 per cent inhibition. Figures in brackets indicate the number of chickens examined.)

Substituents	Dose (mg/kg)	Inhibition %
A. Tri-3-Me/tri-4-Me (60/40)	1000	8 (2)
Tri-2:6-di-Me	500	18 (2)
Tri-3:5-di-Me	500	24 (3)
Tri-3-Me	500	3 (1)
Tri-4-Me	1000–500	21 (3)
Tri-2-Cl	1000	4 (2)
B. None	500	60 (4)
Tri-3-Et	500–1000	69 (2)
Tri-2:3-di-Me	500	28 (2)
Tri-3:4-di-Me	500	37 (2)
Tri-2-Me-4-Et	500	38 (2)
C. Tri-2-Me	500	83 (7)
Tri-2-Et	200–1000	81 (4)
Tri-2-*n*-Pro	200–1000	76 (4)
Tri-2:5-di-Me	500	80 (4)

TABLE 6. THE EFFECT OF ASYMMETRICAL TRI(SUBSTITUTED)PHENYLPHOSPHATES ON CHICKEN BLOOD CHOLINESTERASE ACTIVITY 24 HR AFTER A SINGLE DOSE BY MOUTH

(Group (1) Combinations from group A (Table 5). Group (2) Combinations from groups A and B (Table 5). Group (3) Combinations from groups A and C (Table 5). Group (4) Combinations from groups B and C (Table 5). Figures in brackets indicate the number of chickens examined.)

Substituents	Dose (mg/kg)	Inhibition %
(1) Mono-4-Et, Di-3:5-di-Me	500	15 (2)
Mono-4-Et, Di-4-Me	500	15 (2)
Mono-4-Me, Di-4-Et	500	14 (2)
(2) Mono-4-Et, Di-3-Et	500	42 (2)
(3) Mono-2-Me, Di-4-Me	500	77 (2)
Mono-2-Me, Di-3:5-di-Me	1000	84 (2)
Mono-2-Et, Di-4-Me	500	91 (2)
Mono-2-*n*-Pro, Di-3:5-di-Me	500	84 (2)
Mono-2-*n*-Pro, Di-4-Et	1000	92 (4)
Mono-4-Me, Di-2-Et	200	86 (2)
Mono-2-Et, Di-3:5-di-Me	500	92 (2)
Mono-4-Me, Di-2-*n*-Pro	500	75 (2)
(4) Mono-3-Me, Di-2-Me	500	87 (2)

Esters with mono-4-alkyl substituted phenols are not inhibitory. On the other hand dimethylphenyl esters with or without 4-substitution showed similar (mild) inhibitory activity (Table 4). The most active tri-dimethylphenyl ester was that with the 2:5-substitution. Although there are some correlations between chemical structure, and cholinesterase inhibitory activity, the exact relationship remains uncertain because the inhibitory activity must depend upon metabolites of the original compounds and the structure of these is at present quite unknown.

4. *Esterase inhibition and neurotoxic action*

It has been known for some years that tri-2-methylphenylphosphate was a very active inhibitor of liver and gut esterases in the rat.[20] Recent work on the potentiation of the organophosphorus compound malathion (O:O-dimethyl-S-diethylsuccinyl-thiophosphonate) has confirmed this in the case of TOCP[21] and some other triaryl phosphates (Casida[22]; A. N. Worden, unpublished). It seemed therefore worth while trying to find out whether this ability to inhibit esterase could be linked with either chemical structure of these aryl phosphates or their neurotoxicity. For this purpose two pairs of compounds in which chemical structure and neurotoxicity were uncorrelated have been studied.

The behaviour of the four compounds against an esterase preparation *in vitro* are given in Table 7. The pair of 2-substituted phenyl phosphates are very much less

TABLE 7. THE INHIBITORY ACTION OF FOUR TRI(SUBSTITUTED)PHENYLPHOSPHATES AGAINST THE ESTERASE HYDROLYSING PHENYL BUTYRATE IN CHICKEN LIVER HOMOGENATE

(All inhibitors were incubated for 15 min at 37 °C prior to the addition of substrate. The mean total esterase activity was 528 μl CO_2/ml of 10 per cent homogenate/min with an A-esterase activity of 114, giving a B-esterase activity of 414. The concentration of inhibitor necessary to produce 50 per cent inhibition was determined graphically using results corrected for A-esterase activity. When a saturated solution of the inhibitor did not produce 50 per cent inhibition, the inhibition obtained is given in brackets. Incubation of chicken plasma cholinesterase for 15 min at 37 °C with 9×10^{-5} M of any of these inhibitors produced no more than 6 per cent inhibition.)

Substituents	Neurotoxicity	Concentration for 50% inhibition
Tri-2-Me	+	1×10^{-4} M (41%)
Tri-4-Me	−	8×10^{-8} M
Tri-2-Et	−	1×10^{-4} M (27%)
Tri-4-Et	+	3×10^{-7} M

active than the 4-substituted analogues but there is no correlation of this activity with neurotoxicity to hens.

When the esterase activity in hens is examined after the compounds have been given by mouth no differences are seen among the four compounds. These observations are set out in Table 8 where the effect on pseudocholinesterase is also noted 24 hr after the dose and neurotoxicity at 10 days. Again, no correlation between biochemical activity and neurotoxic action can be seen but it is interesting to note that the tri(4-substituted

phenyl) phosphates show a specific action against phenylbutyrate esterase that they do not have against plasma (pseudo) cholinesterase.

DISCUSSION

The initial purpose of this study was to confirm the findings of Bondy *et al.*[6] on the neurotoxicity or otherwise of a series of symmetrical and unsymmetrical tri(substituted phenyl) phosphates many of which had not been previously examined. We agree with

TABLE 8. THE ACTIVITY OF THE LIVER AND INTESTINAL ESTERASE HYDROLYSIS OF PHENYL BUTYRATE IN HENS 24 HR AND 10–15 DAYS AFTER GIVING A SINGLE DOSE OF FOUR TRI(SUBSTITUTED)PHENYLPHOSPHATES

(The effect on plasma cholinesterase and the neurotoxicity of each compound is also recorded for comparison. Dose: 500 mg/kg by mouth.)

		Esterase activity (μl CO_2/mg protein per hr)			
		2-Me	4-Me	2-Et	4-Et
Liver esterase	Control 25·5 ± 13·6 (7)				
	24 hr	1·5	1·5	2·7	1·2
	10 days	30·6	4·1	23·3	24·5
Intestinal esterase	Control 20·5 ± 7·4 (6)				
	24 hr	0·44	2·2	0·7	1·5
	10 days	17·0	12·9	22·1	23·3
Plasma ChE % inhibition	24 hr	78	5	81	21
Neurotoxicity	10 days	+	−	−	+

those authors in being unable to find an overall correlation between chemical structure and neurotoxicity. Among small groups of compounds some rules appear to emerge. Thus compounds containing one, two or three 2-methylphenyl residues are toxic. The findings of others with different compounds containing these groups confirm this generalization.[2, 4] However, other substituents affect the toxicity in so far as mono-2-methylphenyl, di-4-methylphenylphosphate is more toxic than mono-2-methylphenyl, di-3:5-dimethylphenylphosphate. This is the only limited generalization that can be made for the series of compounds studied here. For instance 2-methyl substitution alone does not confer neurotoxicity for tri-2:3-, tri-2:5- and tri-2:6-dimethylphenyl-phosphates are not toxic; it appears therefore that the presence of a 2-methylphenyl residue is the essential feature. No similar generalization may be made for the presence of 2-ethylphenyl or 2-*n*-propylphenyl residues and in addition the neurotoxicity of tri-4-ethylphenylphosphate[6] is completely unexpected.

Metabolism of these compounds may account for the lack of correlation between structure of the administered compounds and their neurotoxicity. Several years ago it was clearly shown that tri-2-methylphenylphosphate was not an inhibitor of pseudo-cholinesterase *in vitro* The inhibition found *in vivo* after its administration indicated that it must be a metabolite causing the inhibition and it was shown that liver slices *in vitro* can produce an inhibitor.[12] This indicated the possibility that the neurotoxicity might be caused by a metabolite instead of the parent compound. The present work does not allow us to decide whether a metabolite or the parent compound is

neurotoxic. However, the experiments so far carried out illustrate some of the difficulties in reaching even a tentative hypothesis.

A major difficulty is knowing whether any metabolic transformation affects one, two or three of the phenyl rings. For example, tri-2-methylphenylphosphate and tri-2-ethylphenylphosphate do not inhibit plasma cholinesterase *in vitro* and have only a low inhibitory activity against the esterase hydrolysing phenylbutyrate. After administration, both cholinesterase and the esterase are inhibited and therefore this inhibition must be due to a metabolite. Although the results in this paper show that only one 2-methylphenyl or 2-ethylphenyl residue is necessary for the production of a cholinesterase inhibitor, nevertheless we have no evidence that the inhibition of cholinesterase and esterase is caused by the same metabolite.

Tri-4-methylphenyl- and tri-4-ethylphenyl-phosphates do not inhibit plasma cholinesterase *in vitro* but are potent inhibitors of esterase. *In vivo* tri-4-methylphenylphosphate produces no inhibition of cholinesterase but caused almost complete inhibition of esterase (phenylbutyrate as substrate) in liver and intestine. These results are entirely consistent with the *in vitro* findings and it is not necessary to postulate metabolic conversion. Tri-4-ethylphenylphosphate likewise causes complete inhibition of esterase but only slight inhibition of plasma cholinesterase. Nevertheless this inhibition of cholinesterase although slight is more than is expected since it is completely inactive *in vitro*. If this is taken at its face value it must be concluded that tri-4-ethylphenylphosphate is also metabolized *in vivo*. However, the question of purity here is vital. Tri-2-ethylphenyl- or tri-3-ethylphenyl phosphates both cause inhibition of cholinesterase *in vivo*. Tri-2-ethylphenylphosphate is particularly active as are compounds with one or two 2-ethylphenyl residues. Consequently a small amount of 2-ethylphenol in the original 4-ethylphenol used for the synthesis would result in this inhibition. With tri-4-methylphenylphosphate impurities are not likely to be present. It is a solid (m.p. 76 °C) and may be readily purified by recrystallization. Tri-2-ethylphenylphosphate is a liquid (b.p. 221–223 °C at 1·0 mm Hg pressure, cf. Table 2). The question whether tri-4-ethylphenylphosphate is metabolized to an inhibitor of cholinesterase in the chicken is therefore unsettled.

In addition there is the further complication that we have no information of the intrinsic activity of the metabolites which are produced and therefore no idea of the amounts of the administered dose which have to be metabolized to produce the observed inhibitions. Therefore although it is clear that many of these triaryl phosphates are metabolized *in vitro*, it must be appreciated that the only criterion used here for metabolism is the demonstration of inhibition of cholinesterase or esterase. Compounds might well be produced which are inactive in this respect. It is therefore a completely open question whether the parent compound or a metabolite is causing the neurotoxicity. It might be thought that the lack of correlation between structure and neurotoxicity was suggestive that a metabolite was more likely to be the active substance. However, it could be that those compounds which produce neurotoxicity are those which are slowly metabolized.

The relation between cholinesterase inhibition and structure of the triaryl phosphate administered is more straightforward. All compounds which produce more than 75 per cent inhibition of cholinesterase after administration of 0·5 g/kg orally contain at least one phenyl residue with the only substituent in the ring in the 2-position. More than one substituent in the ring often lowers the inhibition obtained as is shown by

tri-2:6-dimethylphenyl- and tri-2:3-dimethylphenyl-phosphate. Further, if we have tri-A-phosphate producing more than 75 per cent inhibition and tri-B-phosphate producing less than 25 per cent inhibition (when A and B are alkyl substituted phenyl residues) then any combination of these residues in one molecule such as mono-A-, di-B- or mono-B-, di-A-phosphate will produce more than 75 per cent inhibition. This generalization has been found to hold for all the compounds studied in this paper. Since all are inactive *in vitro* as inhibitors of cholinesterase, they must be metabolized *in vivo*. This correlation must indicate that metabolism of the group associated with high inhibition (A groups) is not affected by the nature of the other phenyl residues (B groups).

Suggestive evidence that the metabolic change might occur at the alkyl group itself is that tri-2-chlorophenylphosphate does not cause any inhibition of plasma cholinesterase after oral administration of 1·0 ml/kg. However, tri-phenylphosphates produce considerable inhibition and this may mean that substitution in the phenyl ring can play a part in converting these compounds to inhibitors of cholinesterase. Myers *et al.*[23] suggested that *p*-hydroxylation was a likely step and this might explain why tri-3-methyl- and tri-4-methylphenyl-phosphates cannot be converted to inhibitors. However, our findings with the tri-dimethylphenylphosphates are not consistent with his hypothesis. The tri-2:6-dimethylphenylphosphates instead of being the most active is inactive while the 2:5-substituted analogue is the most active and the 3:4 is more active than the 2:6.

Recent work has revealed a further series of phosphorofluoridates which produce the same signs of neurotoxic damage as tri-2-methylphenylphosphate[7] though in doses of one-hundredth or less of those required for the triaryl phosphates. No hypothesis of the mechanism of this lesion can be seriously entertained unless it takes into account these facts. The only common link between these two groups is phosphorus, but of course many tri-esters of phosphoric acid are not neurotoxic. Even in a closed series, however, no link between the two groups of compounds has been found. For instance although di-*n*-propylphosphorofluoridate is neurotoxic at 0·25 mg/kg[7] and tri-2-methylphenylphosphate at 250 mg/kg, di-*n*-propoxy-mono-2-methylphenylphosphate is not neurotoxic in doses up to 1000 mg/kg.

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PHOSPHORYLASE ACTIVITY IN HEART AND BRAIN AFTER RESERPINE, IPRONIAZID AND OTHER DRUGS AFFECTING THE CENTRAL NERVOUS SYSTEM*

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Abstract—Phosphorylase activity in heart and brain was determined after treatment of intact rats with several agents affecting the central nervous system. Reserpine decreases the percentage of phosphorylase *a* in the heart. There is suggestive but non-conclusive evidence that, under certain conditions, reserpine may also lower the phosphorylase *a* content of the brain. Iproniazid pretreatment does not prevent, and, in fact, may enhance the diminution of phosphorylase *a* in the heart and brain of reserpinized animals. Iproniazid alone produces small but non-significant increases of phosphorylase *a* in heart and brain. Serotonin, chlorpromazine and pentobarbital have no effect on brain or heart phosphorylase *a*.

ALTHOUGH many studies have been made of phosphorylase in skeletal muscle,[1–3] relatively little is known concerning this enzyme in cardiac tissue. Phosphorylase is the enzyme which catalyzes the reaction: glycogen + inorganic phosphate ⇋ glucose-1-phosphate.[4] It has been obtained from skeletal muscle in two forms; as a euglobulin form *a* (active) which has 60–70 per cent of its full activity without addition of adenylic acid and as form *b* (inactive) which requires the presence of adenylic acid as co-factor to elicit activity.[5] In skeletal muscle, the predominant phosphorylase is the *b* form.[6]

Following a report on the purification of phosphorylase from dog heart, in which the enzyme exists in both an active and an inactive form,[7] and the observation on the high phosphorylase *a* content of heart muscle,[2] more recent studies have appeared which indicate that functional activity, drugs and hormones may affect phosphorylase activity in the heart. Among the several observations, there is complete agreement that epinephrine and other inotropic catecholamines increase phosphorylase *a* content in cardiac tissue[8–12] possibly through their action in promoting the formation of an essential cyclic nucleotide which is necessary for phosphorylase activation.[13]

Inasmuch as phosphorylase *a* levels may be dependent on intrinsic catecholamines, it would be interesting to study the effects of reserpine on heart phosphorylase *a*, since that agent is capable of nearly depleting cardiac catecholamines.[14] A recent report indicates that, concomitant with a negative chronotropic action, there is no change in phosphorylase *a* in the isolated guinea pig atrium treated with reserpine.[15]

Not only should heart be subject to such studies, but also brain, wherein reserpine causes a release of catecholamines.[16] The investigations on brain would also provide quantitative control data on phosphorylase *a* which, to our knowledge, have not been reported for that tissue.

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For comparison with reserpine, experiments were also performed with several other agents affecting the central nervous system.

METHOD

After appropriate intraperitoneal treatment (see tables), male Wistar strain rats weighing approximately 300 g were decapitated. The head was placed immediately in liquid nitrogen, as was the rapidly excised whole heart. To remove brain cortex, the frozen head was sagittaly split and cortical material chipped out with a carpenter's chisel. The tissue was weighed in the frozen state, then placed in ice-cold medium containing 0·001 M Versene and 0·02 M NaF, and immediately homogenized. The supernatant fraction from the homogenate was assayed.

The phosphorylase assay

The method of Cori and Illingworth[17] was employed, as in previous studies.[9, 12] In brief, the determination of phosphorylase *a* and *b* in mixtures is based on two parallel activity measurements, one without and one with the addition of adenylic acid. In the former case, phosphorylase *a* shows on the average 65 per cent of its full activity, while phosphorylase *b* is inactive; in the latter case, phosphorylase *a* and *b* show full activity.

Phosphorylase activity is measured in the direction of glycogen synthesis by determination of the amount of inorganic phosphate formed from glucose-1-phosphate. Results are expressed as μmoles of inorganic phosphate formed and the ratio:

$$\frac{\text{inorganic P formed without adenylic acid}}{\text{inorganic P formed with adenylic acid}} \times 100$$

is designated in this paper as per cent of active phosphorylase *a*.

Effect of freezing tissue samples. It has been reported that freezing in a CO_2–ethanol slush may cause a breakdown of ATP and consequent carry over of significant amounts of adenylic acid into the assay, giving rise to artifactually high phosphorylase *a* values.[10] In a previous study carried out in this laboratory,[12] it was found that the phosphorylase *a* activity of rat atria which had not been frozen was in the same range as that reported for rat ventricles which had been frozen with a CO_2–ethanol slush. Furthermore, in the present study a comparison was made between the phosphorylase *a* content of rat ventricles (5) which had not been frozen, with samples which had been frozen in liquid nitrogen. The two types of treatment did not result in phosphorylase *a* values which were appreciably different. In the case of brain, there was some delay in the removal of non-frozen tissue, and a comparison of frozen tissue with fresh tissue was not made. Brain and heart tissue samples which had been diluted 1 : 160 were compared with samples which were diluted 1 : 40, as in our standard procedure; there was no marked difference in their respective phosphorylase *a* activity. This would suggest that the amount of endogenous adenylic acid present was not great enough to influence the results.

Centrifugation. Heart and brain homogenates were centrifuged for 15 min at 900 *g* at 0 °C. Preliminary studies indicated that this centrifugation did not result in an appreciable loss of phosphorylase with the discarded precipitate.

Phosphatase controls. Four determinations each, on brain and heart, in which glycogen primer was omitted from the assay system, indicated that phosphatase activity made negligible contributions to inorganic phosphate release. No corrections were

applied since these were sufficiently minor as not to change the nature or the interpretation of the data. Under certain conditions, otherwise negligible but constant corrections may tend to lower phosphorylase *a* values; thus, it is considered advisable to check this point in each investigation.

TABLE 1. PHOSPHORYLASE ACTIVITY AFTER ADMINISTRATION OF RESERPINE TO RATS
(The phosphorylase assay system contained the following in a total volume of 0·2 ml: 5 mg of tissue, 1% glycogen, 0·016 M glucose-1-phosphate and 0·001 M adenylic acid when required. The mixture was incubated for 5 min at 30 °C.)

Controls (reserpine solvent alone)			Reserpine treated (0·8 mg/kg per day for 7 days)		
μmoles of P liberated without adenylic acid: phosphorylase *a*	μmoles of P liberated with adenylic acid: total phosphorylase *a* + *b*	% phosphorylase *a*: $\frac{a}{a+b} \times 100$	μmoles of P liberated without adenylic acid: phosphorylase *a*	μmoles of P liberated with adenylic acid: total phosphorylase *a* + *b*	% phosphorylase *a*: $\frac{a}{a+b} \times 100$
			Brain		
0·105	0·172	61·0	0·142	0·262	54·2
0·141	0·171	82·5	0·136	0·176	77·3
0·110	0·190	57·9	0·089	0·182	48·9
0·177	0·232	76·3	0·137	0·254	53·9
0·177	0·222	79·7	0·129	0·178	72·5
0·143	0·198	72·2	0·110	0·160	68·8
0·185	0·222	83·3	0·112	0·176	63·6
0·129	0·197	65·5	0·121	0·194	62·4
0·127	0·163	77·9	0·083	0·162	51·2
0·107	0·145	73·8	0·099	0·155	63·9
Mean = 0·140	Mean = 0·191	Mean = 73·0 ± 2·8	Mean = 0·116	Mean = 0·190	Mean = 61·7 ± 2·3
Decrease in % phosphorylase *a* in brain after reserpine: 11; $t = 2{\cdot}9$; $P = 0{\cdot}01$					
			Heart		
0·574	0·755	76·0	0·512	0·784	65·3
0·456	0·651	70·0	0·532	0·776	68·6
0·444	0·615	72·2	0·375	0·693	54·1
0·352	0·516	68·2	0·350	0·527	66·4
0·377	0·565	66·7	0·383	0·572	67·0
0·416	0·661	62·9	0·346	0·612	56·5
0·383	0·535	71·6	0·312	0·601	51·9
0·471	0·626	75·2	0·246	0·418	58·9
Mean = 0·434	Mean = 0·616	Mean = 70·4 ± 1·5	Mean = 0·382	Mean = 0·623	Mean = 61·1 ± 2·3
Decrease in % phosphorylase *a* in heart after reserpine: 9; $t = 3{\cdot}2$; $P < 0{\cdot}01$					

RESULTS

In the summer of 1959 the first experiments were performed on heart and brain employing reserpine. The results are shown in Table 1.

It appeared that reserpine effectively lowered the percentage of phosphorylase *a* in both heart and brain. Noteworthy is the fact that brain, as well as heart, possesses a high initial level of phosphorylase *a*.

In the fall of 1959 and winter of 1960 further experiments were performed with other agents affecting the central nervous system. These were carried out in a randomized fashion and a new series of controls was included. Near completion of the studies it was noted that the new controls for the heart showed values almost exactly those of the earlier controls. However, the brain levels were now several per cent

lower. Time-to-time variations in control observations have been a cause for concern in the past but no definitive explanations have been offered for their occurrence.[9, 12, 18] In any event, if the new brain controls were to be compared to the reserpine-treated animals of the old series, the results would be rendered insignificant; accordingly, a new series of observations on the effect of reserpine was included. The results of all studies in the new series are presented in Table 2.

TABLE 2. PHOSPHORYLASE ACTIVITY AFTER VARIOUS DRUG TREATMENTS

Drug	Mean % phosphorylase *a* ± s.e. Brain	Heart
Control; $n = 16$	64·2 ± 1·8	68·5 ± 1·2
Reserpine PO_4 1·0 mg/kg per day for 7 days; (2nd series); $n = 9$	61·1 ± 2·1	54·7 ± 2·4*
Iproniazid PO_4 25 mg/kg per day and reserpine PO_4 1·0 mg/kg per day given simultaneously for 7 days; $n = 6$	61·0 ± 2·8	55·7 ± 3·3*
Reserpine PO_4 10 mg/kg; after 19–26 hr; $n = 8$	62·0 ± 3·8	54·5 ± 1·8*
Iproniazid PO_4 175 mg/kg given 17–22 hr prior to reserpine PO_4 10 mg/kg; $n = 8$	56·2 ± 3·4	47·0 ± 3·4*
Iproniazid PO_4 100 mg/kg; after 17–22 hr; $n = 6$	69·2 ± 5·0	73·9 ± 3·3
Iproniazid PO_4 250 mg/kg; after 17–22 hs; $n = 5$	66·0 ± 1·5	73·2 ± 2·7
Serotonin creatinine SO_4 20 mg/kg; after ½ hr; $n = 8$	60·1 ± 2·3	71·6 ± 2·6;
Iproniazid PO_4 175 mg/kg given 17–22 hr prior to serotonin creatinine SO_4 20 mg/kg; $n = 8$	61·4 ± 2·1	68·1 ± 2·6
Chlorpromazine 1 hr after 2nd of two doses, 25 mg/kg each, given 17–25 hr apart; $n = 8$	67·2 ± 3·1	69·9 ± 3·3
Pentobarbital 50 mg/kg; after 1 hr; $n = 8$	59·9 ± 2·1	65·8 ± 2·3

* Significant at the 1% level. Test for significance according to: C. W. Dunnett, A multiple comparison procedure for comparing several treatments with a control. *J. Amer. Statist. Ass.* **50**, 1096 (1955).

Once again, chronic reserpine administration was shown to lower the phosphorylase *a* content of the heart, but it appeared that the earlier result with brain was not reproducible. Further, it is seen that single high doses of reserpine also had significant actions in lowering active enzyme in the heart but, here too, the brain effect was not significant.

In regard to other aspects of the study, it was noted that the concurrent administration of or pretreatment with iproniazid did not inhibit the ability of reserpine to lower phosphorylase *a* in the heart. In the rats given reserpine after pretreatment with iproniazid, brain phosphorylase *a* showed a downward trend to a point close to the 5 per cent level of probability. Iproniazid alone produced slight rises which were not significant.

It appeared that serotonin, chlorpromazine and pentobarbital had no effect on the phosphorylase *a* content of heart and brain.

DISCUSSION

One unequivocal positive observation has been noted as a result of the present study. Reserpine significantly lowers the phosphorylase *a* content of the heart of the

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rat. This contrasts with the study which reports that reserpine has no effect on phosphorylase *a* in the isolated guinea pig atrium,[15] but correlates well with the observation that reserpinized hearts show an increase of glycogen,[19] a finding which one might expect if glycogenolysis is impaired through an enzyme defect.

Our results with brain are equivocal; the earlier series of experiments indicated significant lowering of phosphorylase *a* in brain upon chronic reserpine administration, but the new series showed no changes. Although a diminution of active enzyme would fit well with the finding that reserpinized animals show an increase in brain glycogen,[20] it should be emphasized that central nervous system effects are noted at a time when phosphorylase *a* is unchanged in the brain of rats treated with single high doses of reserpine; thus, there seems to be no correlation between enzyme activity and the central nervous system action.

The experiments in which iproniazid was given alone were performed on the supposition that a converse result to that obtained with reserpine might be expected. The former agent causes an increase in catecholamine levels in brain.[21] The resultant rise might then lead to increases in active enzyme. However, in regard to brain, it should be noted that, thus far, catecholamines have not been implicated in promoting cyclic nucleotide formation in that tissue.[22]

It was observed that iproniazid, of all agents studied, tended to produce increases in phosphorylase *a* in both heart and brain, but these rises were not significant. This lack of significance would not be surprising, even in the best of situations, since at our control levels of 65 per cent, phosphorylase *a* is already at, or close to, maximum activity.[5] Consequently, any agent which may potentially show increases would be operating against this limitation. Further, a negative result in heart is not unexpected, since rat hearts show only a non-significant 15 per cent rise in catecholamines after iproniazid.[23] Thus, our iproniazid results are inconclusive in that we are uncertain as to whether the lack of significance is real or apparent.

Through a mechanism that is not clear, animals pretreated with iproniazid before reserpine administration are reported to show considerably less of a loss in adrenal, brain[21] and heart[24] catecholamines than if they were treated with reserpine alone. If lowering of phosphorylase *a* is due to impairment of the activation of that enzyme, as a result of reserpine-induced amine depletion, one may test whether iproniazid may prevent the effect of reserpine on phosphorylase *a*.

Our experiments on heart and brain show that iproniazid-pretreatment, or iproniazid given concurrently with reserpine, does not have any effect in preventing the diminution of active enzyme that obtains when reserpine is given alone, particularly in the case of the heart. Unless it is contended that iproniazid does not afford adequate protection, as indeed may not occur in the rat heart,[23] this finding suggests that the effect of reserpine on phosphorylase *a* may not be dependent on catecholamines and, thus, direct or other mechanisms cannot be ruled out.

One interesting observation which in no way resolves our previous dilemma concerning phosphorylase activity in the brain after reserpine administration is that, in the iproniazid-pretreated animals, reserpine produces a downward trend in phosphorylase *a* in brain to an extent that places the result at the borderline of the 5 per cent level of probability. In the heart too, it appears that active enzyme is diminished to an even greater degree after reserpine in the iproniazid-pretreated rats. The significance of this finding is not apparent.

Our study indicates that serotonin, chlorpromazine and pentobarbital have no effect on phosphorylase *a* in brain or heart and, for the present, support the specificity of action of reserpine where that effect is of a positive nature.

It is difficult to assess the therapeutic significance of our findings relating to the definite action of reserpine in lowering phosphorylase *a* in the intact heart. Concern has already been voiced regarding potential dangers with any agent which decreases the amount of the intrinsic catecholamines in the heart.[25] Now, whether it be indirectly through catecholamine depletion or via direct action, we are faced with a significant alteration of an enzyme activity in heart upon reserpine administration. Not until the role of phosphorylase in cardiac metabolism is evaluated can we estimate the importance of the observation.

Addendum. While this work was in progress, an abstract by M. E. Hess, J. Shanfeld and N. Haugaard appeared in *Fed. Proc.* **19**, 108 (1960); in this the action of reserpine in decreasing phosphorylase *a* in the intact rat heart was noted. No data were provided. On the other hand, a still later (July) paper, by S. E. Mayer and N. C. Moran, *J. Pharmacol.* **129**, 271 (1960), indicated that neither reserpine alone nor serotonin had any effect on phosphorylase *a* in the intact dog heart. In June, S. L. Leonard and H. T. Day reported, in *Proc. Soc. Exp. Biol. Med., N.Y.* **104**, 338 (1960), that serotonin had no effect on phosphorylase *a* in rat heart.

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EFFECTS OF ACETYLCHOLINE ANALOGUES AND ETHANOL ON THE RESPIRATION OF BRAIN CORTEX TISSUE *IN VITRO*

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Abstract—Unstimulated and electrically stimulated brain cortex slices were exposed to compounds related to acetylcholine, and to Triton, with or without $1{\cdot}96 \times 10^{-1}$ M (0·9 per cent) ethanol.

The lipid-insoluble substances acetylcholine, curare, deca- and suxa-methonium were inactive on the brain tissue respiration. In unstimulated tissue, the lipid-soluble acetylcholine analogues pyridine-2-aldoximedodecyl iodide (PAD) and cetyltrimethylammonium bromide (Cetavlon), like ethanol, caused a transient increase in respiration. Cetavlon, cetylpyridine chloride (CPC) and Triton reduced the respiration of both stimulated and unstimulated tissue at approximately identical concentrations, the relative effect on unstimulated tissue being smaller. 10^{-4} M PAD and $1{\cdot}8 \times 10^{-3}$ M atropine abolished the response to stimulation almost completely leaving unstimulated tissue unaffected. Ethanol significantly increased the effect of atropine, PAD, Cetavlon and CPC on stimulated tissue. The non-ionized detergent Triton was not synergistic with ethanol.

In intact rats, PAD potentiated ethanol action on the CNS Cetavlon acted similarly, but caused strong haemolysis when injected with ethanol. *In vitro*, haemolysis caused by Cetavlon was not increased by low ethanol concentrations.

The results are consistent with the hypothesis that the acetylcholine system is involved in the reaction of brain slices to electrical stimulation, and indicate that ethanol may depress the functional response to stimulation by unspecific action on this system.

ETHANOL at concentrations associated with intoxication *in vivo* increases the respiration of unstimulated brain slices at least transiently[1, 2] and depresses that of brain tissue stimulated with potassium ions[2, 3] or with applied electrical pulses.[3, 4] As stated by Hunter and Lowry,[5] in seeking an explanation for the mechanism of anaesthesia, the crucial problem is perhaps to decide between two alternative possibilities: inhibition of a sensitive energy-yielding reaction essential for the functioning of nerves, or interference with specific transmission mechanisms. Work in these laboratories[3, 4] and by Beer and Quastel[6, 7] with brain cortex slices, and electrophysiological and metabolic evidence obtained with peripheral nerves,[8–11] has led the author tentatively to adopt the view that the depression of respiration in stimulated brain tissue exposed to ethanol is a secondary effect due to inhibition of the functional response. Wallgren and Kulonen[3] have also suggested that the effect of ethanol in increasing the respiration of unstimulated tissue may be due to its well established depolarizing action.

Rowsell[11a] has demonstrated that combined acetylcholine is lost from cerebral cortex slices during electrical stimulation. During recent years, Nachmansohn's theory of the essential role of the acetylcholine system in the excitation cycle of

conducting membranes (cf. the recent monograph[12]) has gained support from experiments with analogues of acetylcholine made lipid-soluble by substitution of one of the methyl groups with a long aliphatic chain. Some of the physiological effects of such a compound (PAD) were first described by Wilson[13], and most of the results obtained thus far have been reviewed.[12] Accepting Nachmansohn's theory and supposing that the neurones are activated in brain slices during electrical stimulation, one would expect lipid-soluble quaternary ammonium ions selectively to depress the respiratory response to stimulation. Furthermore, such compounds would be expected to cause at least a transient increase in the respiration of unstimulated slices. In the present investigation, these problems were studied, and also the relationship between the effect of ethanol and any changes possibly induced by the quaternary ammonium compounds.

Some of the compounds used were strong detergents, and their effects on brain tissue were compared with those of a non-ionized detergent. Haemolysis was used as a simple test of surface activity in a biological system and the results contrasted with those obtained with brain tissue in order to distinguish unspecific surface action from effects on systems specifically related to the function of nerve tissue.

MATERIALS AND METHODS

Compounds tested

Acetylcholine chloride (Hoffmann–La Roche); decamethonium iodide (L. Light & Co.); suxamethonium iodide grade C (Cal. Corp. Biochem. Res.); D-tubocurarine (Theodor Schuchardt, G.m.b.H.); pyridine-2-aldoximedodecyl iodide (PAD, obtained through the courtesy of a local laboratory); pyridine-2-aldoximemethyl iodide (PAM, Dajac); cetylpyridine chloride (CPC, AS Bionova); cetyltrimethylammonium bromide (Cetavlon, Fluka AG.); the alkylarylpolyethoxy alcohol Triton X-100 (Rohm & Haas Co.); racemic atropine sulphate (Bidle Sawyer & Co.); eserine sulphate (C. F. Boehringer & Soehne G.m.b.H.).

Experiments with brain cortex slices

Apparatus. All experiments were performed with conventional Warburg constant-volume respiratory manometers. A detailed description of the stimulators and electrode flasks has been given previously.[3] The only change in the equipment was that the earlier vibrators (with the frequency 90–100 c/s) were exchanged for new ones (Philips 7934/02; with the frequency 50 c/s) giving a pulse frequency of 100/sec. However, neither the stability nor the magnitude of the response was altered. A voltage gradient of 1·5–1·6 V/mm as measured with an electronic voltmeter was maintained between the electrodes.

Experimental conditions. Phosphate buffered medium[14] with 6 mM glucose was used throughout, the volume per flask being 2·5 ml. The gas phase was pure oxygen and the experimental temperature 37·5 °C.

Adult rats of the laboratory stock were used. The preparation of the slices and the general procedure have been described.[3] All inhibitors were dissolved in the experimental medium, and the solutions were placed in the side-arm of the flasks after gassing.

In some of the experiments, respiration was measured during a 20-min initial control period, after which the inhibitors were tipped into the main vessel and the recording of respiration continued from 25 to 85 min after the first reading. Any change in

respiration relative to the first 20-min period was thus observed. In other experiments, the inhibitors were added during the 5-min period between initiation of stimulation and commencement of manometer reading. The manometers were read at 10-min intervals.

The experiments were usually set up with two standard flasks and two electrode flasks, although preliminary experiments were often made with unstimulated tissue only. When pulses were applied, one slice (20–40 mg) from each brain hemisphere was used for measuring respiration of unstimulated tissue, and two slices (30–50 mg) for that of stimulated tissue. The two pairs of flasks used in each experiment were thus provided with slices obtained from different lobes of the same brain and subjected to different conditions with respect to the inhibitor present.

All compounds were tested in conjunction with ethanol: these experiments were always performed with one pair of flasks with the inhibitor only and the other pair with the same concentration of the inhibitor in the presence of $1{\cdot}96 \times 10^{-1}$ M (0·9 per cent) ethanol. Our earlier experiments with ethanol[3, 4] were supplemented by measuring the effects of higher concentrations of ethanol.

Experiments with intact animals

The effect of PAD, PAM and Cetavlon on ethanol intoxication in rats was measured by means of the "tilted plane" test developed in these laboratories.[15] In testing, the animal is placed, facing up the slope, on an inclined plane with a rough surface, the plane is tilted and the angle at which the animal slides down is recorded. The results are expressed as percentages of an initial sober value obtained immediately before the administration of alcohol.

The animals were fasted overnight and the alcohol was injected intraperitoneally as a 10 per cent (w/v) solution in saline. PAD, PAM and Cetavlon, respectively, were dissolved in this solution when given together with ethanol. When given alone, PAM and Cetavlon were dissolved in saline, PAD in propylene glycol. Propylene glycol in the dose employed (2·5 ml/kg) did not affect the performance of the rats.

In each series of experiments, the animals were of the same age and sex.

Experiments on haemolysis

The haemolytic effect of Cetavlon and Triton on whole blood and on washed red cells was measured in the absence and presence of ethanol. The experiments were carried out at room temperature. The compounds tested were dissolved in the experimental medium used for the brain slices. This was also used for washing and suspending red cells. In experiments with whole blood, the amount of medium and detergent added was 4 per cent of the total volume. The compounds tested were allowed to act on the blood cells for 20 min, including the centrifugation time. The colour developed was measured with a spectrophotometer at 540 mμ.

RESULTS

Respiration of brain slices

In Table 1, the results of experiments with a 20-min control period and subsequent measurement of the drug effects are summarized. For the active compounds, the lowest concentration with a clear depressing effect on the respiration of stimulated tissue is given. The physiologically highly active but lipid-insoluble quaternary ammonium compounds acetylcholine, curare, deca- and suxa-methonium were inactive

in the conditions used. By contrast, Cetavlon and CPC, both containing a long aliphatic chain, depressed respiration at very low concentrations. In addition Cetavlon caused a transient increase in the respiration of unstimulated tissue, an effect which was not found with CPC.

TABLE 1. EFFECTS OF VARIOUS COMPOUNDS ON OXYGEN CONSUMPTION OF BRAIN SLICES IN THE EXPERIMENTS WITH AN INITIAL CONTROL PERIOD

The symbols indicate: (a) no effect; (b) depression; (c) response to stimulation completely abolished; (d) transient increase; (O) no modification of the effect in presence of ethanol; X, depression increased in presence of ethanol.

Compound	Concentration range tested (M)	Effect on oxygen consumption		Synergism with ethanol
		Stimulated tissue	Unstimulated tissue	
Acetylcholine	$1{\cdot}1 \times 10^{-2}$	(a)	(a)	(O)
Eserine	$3{\cdot}1 \times 10^{-5}$–$1{\cdot}5 \times 10^{-3}$	(a)	(a)	(O)
Acetylcholine + eserine	$1{\cdot}1 \times 10^{-2}$ + $3{\cdot}1 \times 10^{-5}$	(a)	(a)	(O)
Curare	$2{\cdot}9 \times 10^{-3}$	(a)	(a)	(O)
Decamethonium	$3{\cdot}9 \times 10^{-5}$–2×10^{-3}	(a)	(a)	(O)
Sux-amethonium	$3{\cdot}9 \times 10^{-5}$–2×10^{-3}	(a)	(a)	(O)
Cetavlon	$5{\cdot}5 \times 10^{-5}$–$1{\cdot}4 \times 10^{-3}$	(b), (c) at $5{\cdot}5 \times 10^{-3}$	(b), (d) at $5{\cdot}5 \times 10^{-3}$	X
CPC	$1{\cdot}5 \times 10^{-5}$–$4{\cdot}7 \times 10^{-4}$	(b), (c) at $4{\cdot}4 \times 10^{-4}$	(b)	X
Triton	15–1000 μg/ml	(b), (c) at 150 μg/ml	(b) from 40 μg/ml	(O)

Since Cetavlon and CPC are strong detergents and their effects might be due to unspecific surface activity, the non-ionized detergent Triton was used for comparison. In the present conditions, the effects were similar to those of CPC. Triton even proved to be rather more selective than Cetavlon or CPC in depressing the respiration of stimulated tissue.

Ethanol clearly augmented the depressing action of Cetavlon and CPC, but not that of Triton. However, the synergism between ethanol and Cetavlon or CPC, respectively, was not very strong, the effects being less than additive. The statistical significance of the increased depression in the presence of ethanol was $P < 0{\cdot}05$ for both compounds.

In 1-hr experiments in which the inhibitor was added immediately after the commencement of stimulation, the tissue respiration was considerably more sensitive to the inhibitor, and there was also a clearer tendency to selective action on the stimulated tissue. Results of 1-hr experiments are presented in Table 2. All the compounds listed had a highly significant depressing action on the respiration of stimulated tissue. Only one, Cetavlon, significantly depressed the respiration of unstimulated tissue ($P < 0{\cdot}01$). As indicated, ethanol increased respiration in the absence of stimulation. The series with ethanol is from an earlier study,[4] but is included here since the oxygen uptake of the previous control series (198 μmoles/g fresh tissue/hr) did not deviate significantly from the present controls. The experiments with Cetavlon and Triton gave results consistent with those obtained earlier, except that Cetavlon had a stronger effect on stimulated tissue (−37 per cent) than on unstimulated tissue (−14 per cent). There was an initial increase in the respiration of unstimulated tissue.

The synergism of ethanol with Cetavlon was highly significant: with Triton, there was again no synergism. Atropine, a tertiary compound, also depressed respiration, but was considerably less effective than Cetavlon. However, with $1{\cdot}8 \times 10^{-3}$ M, there was no response to stimulation, whereas unstimulated tissue did not react to the presence of atropine. Ethanol was clearly synergistic with atropine.

TABLE 2. OXYGEN CONSUMPTION OF RAT BRAIN CORTEX SLICES IN THE 1-HR EXPERIMENTS
The oxygen consumption is expressed as μmoles/g fresh tissue/hr $\pm$ standard deviation. $P <$ shows P-values for significance of the action of ethanol. Control and ethanol refer to different experimental series for which statistical evaluation was performed by means of Student's t-test. In all other series, the two hemispheres of the same brain were used in parallel experiments with identical concentrations of inhibitor, but the tissue from one of the hemispheres was exposed to ethanol, that from the other was not. The significance of the effect of ethanol was calculated by means of the t-test for correlated measures.

Experimental conditions	Stimulated tissue			Unstimulated tissue		
	No. of expts.	Mean oxygen consumption	$P <$	No. of expts.	Mean oxygen consumption	$P <$
Control	21	204 ± 17		31	125 ± 16	
Ethanol $1{\cdot}96 \times 10^{-1}$ M	21	167 ± 12	0·001	21	139 ± 15	0·005
Cetavlon $1{\cdot}1 \times 10^{-4}$ M	9	129 ± 16		9	107 ± 15	Not
Cetavlon + ethanol	8	105 ± 12	0·001	9	95 ± 19	sig.
Triton 15 μg/ml	9	141 ± 12	Not	9	119 ± 9	Not
Triton + ethanol	8	135 ± 6	sig.	9	114 ± 8	sig.
Atropine $6{\cdot}9 \times 10^{-4}$ M	6	162 ± 8		6	115 ± 12	Not
Atropine + ethanol	6	142 ± 20	0·005	6	122 ± 8	sig.
PAD $2{\cdot}4 \times 10^{-5}$ M	10	180 ± 8		11	125 ± 11	Not
PAD + ethanol	10	156 ± 12	0·005	11	117 ± 16	sig.
PAD $4{\cdot}8 \times 10^{-5}$ M	8	165 ± 6		10	128 ± 13	Not
PAD + ethanol	8	140 ± 16	0·025	10	125 ± 19	sig.

The most important results were those obtained with PAD, which even at a very low concentration markedly depressed the respiration of stimulated tissue, whereas that of unstimulated tissue was unaffected. PAD at $4{\cdot}8 \times 10^{-5}$ M was about as effective as ethanol at $1{\cdot}96 \times 10^{-1}$ M. PAD was also synergistic with ethanol in stimulated tissue, the effects being almost additive. The difference between the series with ethanol alone and with $2{\cdot}4 \times 10^{-5}$ M PAD + ethanol appeared rather small, but it was statistically significant ($P < 0{\cdot}05$). The finding with the higher concentration corroborated this point.

In Fig. 1, the effect of increasing concentrations of PAD on respiration is shown graphically. At $9{\cdot}6 \times 10^{-5}$ M PAD, the respiratory reaction to stimulation had almost disappeared whereas the respiration of unstimulated tissue was scarcely affected. However, the respiratory response to stimulation was never immediately abolished: it was noticeable during the first 20 min of stimulation even with the highest concentrations of PAD employed. In Fig. 2, the effect on respiration of varying concentrations of ethanol is presented. The graphs for PAD and ethanol are very similar: however, PAD is about 4000 times more effective than ethanol as an inhibitor of respiratory response to stimulation. As with PAD, there was an initial weak response to stimulation also with the highest concentration of ethanol used.

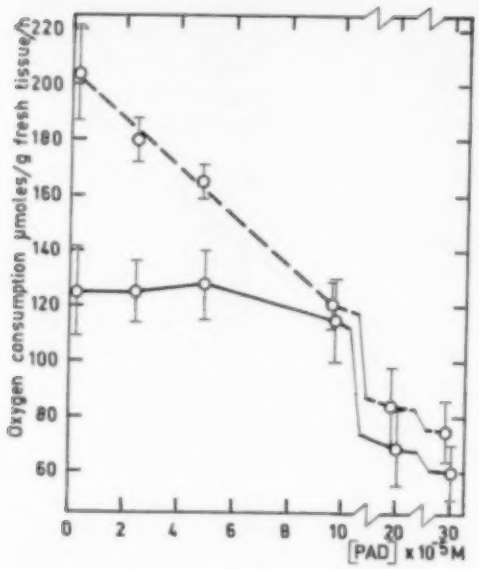

FIG. 1. Effect of PAD on respiration of brain cortex slices. Stimulated tissue (– – –), unstimulated tissue (———). The data in Table 2 concerning controls and PAD are included, the other points represent five determinations each. Vertical bars indicate standard deviation.

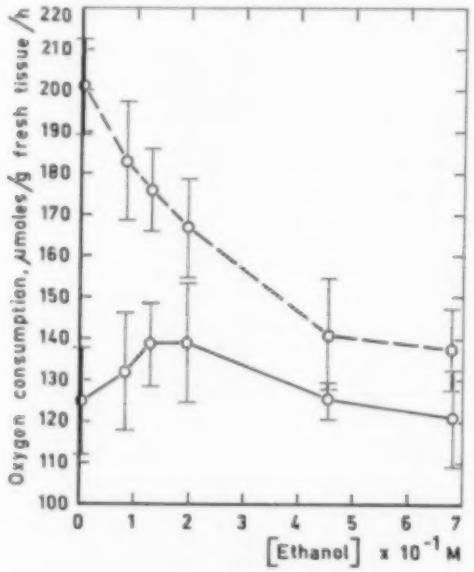

FIG. 2. Effect of ethanol on respiration of brain cortex slices. Stimulated tissue (– – –), unstimulated tissue (———). Each point represents the average of at least ten determinations. Vertical bars indicate standard deviations.

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In Fig. 3, the time course of the oxygen consumption in various conditions is presented. When PAD was added to unstimulated tissue, the respiration rose initially well above and later fell slightly below that of the controls. This was a parallel to the observations with Cetavlon. With ethanol the respiration was elevated throughout the experimental period of 1 hr. However, when ethanol was added after 20 min,

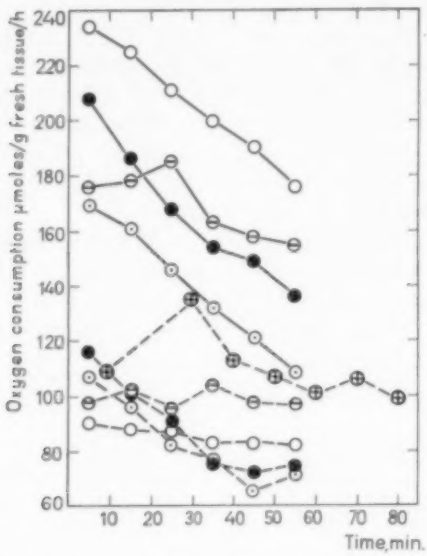

FIG. 3. Time course of oxygen consumption of brain cortex slices. Stimulated tissue (———), unstimulated tissue (– – –). Control medium (○); $4{\cdot}8 \times 10^{-5}$ M PAD (●); $1{\cdot}96 \times 10^{-1}$ M ethanol (⊖); PAD + ethanol (⊙); unstimulated tissue exposed to ethanol after initial 20-min control period (⊕) To avoid overlapping, the ordinates for 1-hr experiments with unstimulated tissue have been reduced by 40 μmoles and those for experiments with initial control period by 30 μmoles. Each curve represents the average of at least eight determinations.

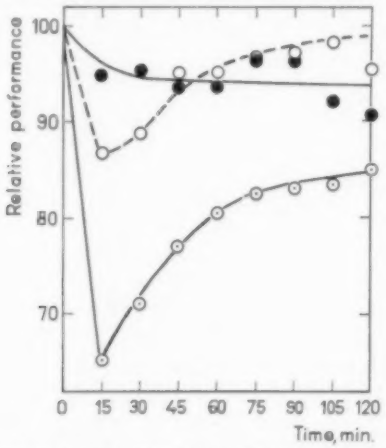

FIG. 4. Time course of intoxication in rats. Ethanol 1·5 g/kg (○); PAD 15 mg/kg (●); ethanol + PAD (○). Each group comprised ten individuals.

respiration increased transiently and then returned to the initial level. PAM in concentrations up to 5×10^{-3} M did not influence respiration.

Intoxication in intact animals

Of thirty female rats, ten received 1·5 g ethanol/kg, ten 15 mg PAD/kg, and ten 1·5 g ethanol + 15 mg PAD/kg in one injection. The time course of intoxication as measured during 2 hr following injection is shown in Fig. 4. The means of the lowest values observed indicated that ethanol reduced performance by 15·5 ± 4·4 per cent, PAD by 11·5 ± 3·7 per cent, and ethanol + PAD by 35 ± 5·8 per cent (results ± standard deviation). PAD thus potentiated the effect of ethanol. With PAD alone, the animals appeared curiously sleepy and depressed, but not inco-ordinated in the same way as with ethanol. PAM in the same molar dose had no observable effects when given alone and did not modify ethanol intoxication.

Cetavlon in a dose of 100 mg/kg approximately doubled the effect of 2 g/kg ethanol during the initial phase of intoxication. The animals treated (five males) became exanotic after about 90 min, and died of haemolysis within 2–2½ hr after injection. Five males injected with the same dose of Cetavlon but no ethanol were not overtly intoxicated and there were only slight signs of haemolysis. No other untoward effects were observed.

Haemolysis

The observation of haemolysis in intact animals treated with Cetavlon and ethanol prompted *in vitro* measurements of the haemolytic effect of Cetavlon. When ethanol

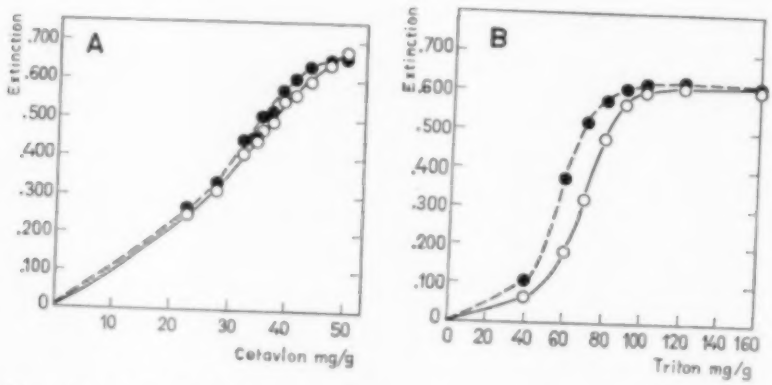

FIG. 5. Haemolysis of washed red cells in presence of (A) Cetavlon, (B) Triton. Cetavlon (Triton) only (○); Cetavlon (Triton) + 0·9 per cent ethanol (●). The curves for Cetavlon are averages of three series of measurements, those for Triton of two series.

was added at a concentration (0·3 per cent) approximately corresponding to the highest concentration to be expected in the blood of the intact animals, no effect on the haemolysis caused by Cetavlon was observable either in whole blood or in washed red cells. Thus ethanol did not directly augment the haemolysis in the intact animals but apparently facilitated the uptake of Cetavlon from the intraperitoneal cavity.

The haemolytic effect of Cetavlon and Triton was then tested *in vitro* with and without addition of 0·9 per cent ethanol in order to obtain effects which could be compared with those observed in brain tissue. The results (Fig. 5) indicate that in this preparation ethanol clearly augmented the effect of Triton, whereas that of Cetavlon was only slightly modified.

DISCUSSION

The lack of effect on respiration of acetylcholine and eserine in the present experiments corroborates the earlier findings of McIlwain[16]. The inactivity of curare, suxamethonium, decamethonium, and PAM is consistent with this observation. In accordance with Nachmansohn, one may assume that acetylcholine and related lipid-insoluble compounds fail to penetrate to the sites of action. These drugs seem to affect conduction at the points of transmission and are known to do so centrally also, for instance after topical application. Perhaps the explanation of the lack of effect on the stimulated slices is that the condensor discharges lead to simultaneous depolarization of all excitable structures without any spreading response.

The most interesting finding with unstimulated tissue is the increase in respiration caused by ethanol, Cetavlon and PAD, all of which are known to be depolarizing agents. It supports the earlier suggestion[3] that depolarization may be the basis of the increased respiration in unstimulated tissue exposed to ethanol. This argument is weakened, however, by the absence of such effects in the presence of CPC. Dodecylpyridine chloride depolarizes[17] and one would accordingly expect CPC to act in the same way. On the other hand, there are exceptions to the general rule that quaternary ammonium compounds have a depolarizing effect.[12]

Atropine also gave results confirming McIlwain's[16] observations. The selective depression of respiration of stimulated tissue by PAD, and also by Cetavlon, constitutes further evidence for the view that the acetylcholine system is involved in the response to stimulation. It also supports the notion that inability to penetrate to the site of action is the reason for inactivity of lipid-insoluble quaternary compounds.

Although the predicted effects were obtained with the lipid-soluble quaternary compounds, this is no unequivocal demonstration of specific action on any of the supposed components of the acetylcholine system. On the basis of analogy with other preparations, however, the results, especially those with PAD and atropine, seem most readily explainable in terms of Nachmansohn's theory. As regards the surface-active compounds, the main argument for a specific action of CPC and Cetavlon in nerve tissue is their marked synergism with ethanol and the absence of such synergism between ethanol and Triton. In the test for haemolysis, the effect of Triton was clearly increased by ethanol, whereas that of Cetavlon was only very slightly altered, thus suggesting that the synergism between ethanol and Cetavlon in brain slices is associated with a process specific to nerve tissue.

Block of conduction by ethanol and by PAD is associated with depolarization. The similarity of their effects on brain tissue is striking. The only differences observed were, firstly the active concentrations, PAD being approximately 4000 times more effective than ethanol, and secondly the difference in the duration of the increase of respiration in unstimulated tissue. The difference in effective concentrations does not prove or disprove specificity of inhibition since it is penetration to the site of action that is decisive.[12] However, no obstacles to the penetration of ethanol would be

anticipated. Especially in view of the synergism on the one hand, and the structural and chemical differences on the other, it does not seem unreasonable to suggest that PAD and ethanol act on the same system, the former in a specific, the latter in an unspecific manner. However, ethanol may act on some structure not directly connected with the acetylcholine system, whereas action on the conducting membranes appears highly likely on account of the synergism with the quaternary ammonium compounds and with atropine.

The fact that the response to stimulation is not completely abolished with either PAD or ethanol may indicate that specific and complete inhibition of the acetylcholine system does not occur, or that operation of the acetylcholine system is not obligatory for the response of the slices to stimulation. The significance of reactions in the residual systems in presence of high concentrations of inhibitors is, however, very difficult to evaluate.

The findings with intact animals indicate that the observed synergism between ethanol and the quaternary ammonium compounds in the brain slices may have relevance for an understanding of alcohol intoxication. Ethanol might be thought to facilitate penetration of PAD in the same way as Cetavlon was more easily absorbed in intact animals when injected together with ethanol. However, the work of Wilson[13] suggests that PAD by itself is readily able to penetrate into brain tissue. Thus the results with the intact animals may reflect the same phenomena as were observed in the brain tissue.

The main conclusions of the present work are thus as follows. Firstly, lipid-soluble quaternary ammonium compounds selectively depress the respiration of brain tissue stimulated electrically as compared with that of unstimulated tissue. This is consistent with the theory that the acetylcholine system is necessary for the functioning of excitable tissue. Secondly, the synergism with ethanol is also restricted to the stimulated preparation. This suggests that ethanol may act directly on processes linked with the excitation cycle of conducting membranes.

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THE MODE OF ACTION OF ALKYLATING AGENTS—II

STUDIES OF THE METABOLISM OF MYLERAN. THE REACTION OF MYLERAN WITH SOME NATURALLY OCCURRING THIOLS *IN VITRO*

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Abstract—Myleran and 1:4-dibromobutane react with L-cysteine at pH 8 to form the S-β-L-alanyltetrahydrothiophenium ion, and smaller quantities of S-di-L-cysteinylbutane and S-(4-hydroxybutyl)-L-cysteine.

The chemical properties of the sulphonium ion are reported: (a) In the presence of aqueous alkali it rapidly decomposes to tetrahydrothiophene and aminoacrylic acid (in tautomeric equilibrium with iminopyruvic acid); (b) Electrolysis yields tetrahydrothiophene and alanine; (c) Thermal decomposition yields mainly tetrahydrothiophene; (d) Virtually no reaction occurs with cysteine at pH 7 or less; (e) At pH 8–9 it reacts rapidly and almost quantitatively with cysteine yielding S-dicysteinylbutane; (f) At pH > 10 in the presence of cysteine preferential reaction occurs with the hydroxyl ion yielding tetrahydrothiophene and aminoacrylic acid.

Myleran reacts with glutathione in water or in aqueous acetone at pH > 8 yielding 30–35 per cent tetrahydrothiophene and bound lanthionine (but no bound S-dicysteinylbutane or S-hydroxybutylcysteine). Possible mechanisms for these reactions are discussed.

Reduced keratin and egg albumin react with Myleran in the presence of alkali to yield tetrahydrothiophene.

Myleran reacts with β-mercaptoethylamine to form the (S-(β-aminoethyl)) tetrahydrothiophenium ion, and with thioglycolic acid in the presence of alkali to yield tetrahydrothiophene.

The possible relevance of these results to the metabolism of Myleran is discussed.

INTRODUCTION

FROM among the bifunctional alkylating agents which have been synthesized and subjected to laboratory and clinical trial 1:4-dimethanesulphonyloxybutane (Myleran), ((I), $n = 4$) has been selected by the majority of clinicians as the most efficacious compound for the palliative treatment of chronic granulocytic leukaemia.[1]

$$CH_3SO_2O(CH_2)_n{\cdot}OSO_2{\cdot}CH_3$$
(I)

In addition to its effects on the blood elements which have been carefully studied by Elson[2], Myleran is a mutagen[3] and has been shown to be carcinogenic in the rat.[4]

It was hoped that identification of the major urinary constituents formed from the alkylating moiety of Myleran after its administration to animals might contribute to the eventual understanding of the chemical mechanism by which it exerts its pharmacological effects. In this connexion the urinary metabolites excreted after injection of

certain monofunctional methanesulphonyloxy esters have already been examined, and earlier publications[5] described the excretion of derivatives of the corresponding S-alkylcysteine after injection of these compounds into the rat. For example 1-^{14}C-ethyl methanesulphonate (II) was shown to be metabolized in the rat to N-acetyl-S-1-^{14}C-ethylcysteine (III) and other derivatives of S-1-^{14}C-ethylcysteine.

$$CH_3SO_2O\cdot\overset{*}{C}H_2\cdot CH_3 \longrightarrow CH_3\cdot\overset{*}{C}H_2S\cdot CH_2\cdot CH(NH_2\cdot CO\cdot CH_3)\cdot COOH$$

(II) (III)

compounds which were considered to have been derived by reaction of the drug, not only with endogenous cysteine, but also with glutathione and thiol compounds of higher molecular weight. It was considered that if Myleran underwent similar reactions with thiol groups *in vivo* the identification of its urinary metabolites might be facilitated by an examination of the reaction products formed by reacting it with cysteine and other thiol-containing compounds *in vitro*.

Preliminary reports of this work have already appeared[6, 7] and subsequently some very relevant findings have been published by Parham and Wilbur[8] on the reaction between Myleran and cysteine-ethyl ester.

MATERIALS AND METHODS

Paper chromatography

The solvents used in the paper chromatography of amino acids were butanol–ethanol–propionic acid–water (20:10:10:4) (solvent I), butanol–acetone–di*cyclo*-hexylamine–water (20:20:10:4) (solvent II), and methylethylketone–acetic acid–water (3:1:1) (solvent III).

Tanks and spray reagents were as previously described.[5]

2:3-^{14}C-Myleran was kindly supplied by Dr. P. Brookes of this Institute.

S-(4-*hydroxybutyl*)-L-*cysteine*

L-Cysteine hydrochloride (3·14 g) in 4 N sodium hydroxide (15 ml) was added rapidly to a hot solution of tetramethylenechlorohydrin (2·13 g) in 50 per cent ethanol (30 ml), and the solution heated under reflux for 0·5 hr. After allowing the mixture to stand overnight at room temperature the precipitated S-(4-hydroxybutyl)-L-cysteine was collected by filtration.

The product crystallized from aqueous acetone as microprisms, m.p. 203° (d.). (Found: C, 44·0; H, 8·0; N, 7·2. Calc. for $C_9H_{15}O_3NS$: C, 43·5; H, 8·0; N, 7·4%.)

S-*β*-L-*alanyltetrahydrothiophenium chloride*

S-(4-hydroxybutyl)-L-cysteine was heated under reflux with an excess of concentrated hydrochloric acid for 0·5 hr. The sulphonium compound formed a picrate which separated from water as small prisms, m.p. 135 °C (d.). (Found: C, 38·3; H, 3·9; N, 13·8; S, 7·5. Calc. for $C_{12}H_{16}O_9N_4S$: C, 38·6; H, 4·0; N, 13·8; S, 7·9%.)

The reaction of Myleran with L-*cysteine*

Myleran (2·46 g) was dissolved in boiling water (20 ml) and treated with a solution of L-cysteine hydrochloride (3·14 g) in 4 N sodium hydroxide (8·4 ml), added dropwise

during 2 min. After heating for a further minute the mixture was quickly chilled in ice and the pH adjusted to 6–7. The solid (0·2 g) which separated was a sulphur-containing amino acid. It was characterized as S-di-L-cysteinylbutane by preparation of its bis-uramino derivative by the general method of Loring and du Vigneaud,[9] plates from a mixture of ethanol, chloroform and petroleum (b.p. 60–80 °C), m.p. 158 °C. (Found: C, 54·1; H, 5·8; N, 9·8; S, 11·7. Calc. for $C_{24}H_{30}O_6N_4S_2$: C, 53·9; H, 5·7; N, 10·5; S, 12·0%.)

The filtrate was treated with a large excess of acetone and the precipitated heavy oil containing salt, cysteine and the sulphonium compound, solidified on standing at 0 °C. Treatment of an aqueous solution with sodium picrate yielded S-β-L-alanyltetrahydrothiophenium picrate, m.p. 135 °C, undepressed on admixture with an authentic sample prepared from S-(4-hydroxybutyl)-L-cysteine as described previously.

Paper chromatography of the mother liquors indicated a variety of ninhydrin positive compounds, one present in relatively high concentration having the same R_f value as S-(4-hydroxybutyl)-L-cysteine in three solvent systems although no further proof of its constitution has been obtained.

The reaction of dibromobutane with cysteine

Dibromobutane (4·32 g) in ethanol (65 ml) and water (30 ml) was treated with a solution of L-cysteine hydrochloride (3·14 g) in 4 N sodium hydroxide (10 ml). The clear solution was allowed to stand at room temperature. Next day a small quantity of S-di-L-cysteinylbutane, characterized by chromatography and as its bisuramino derivative, which had separated out, was removed by filtration. Careful addition of acetone (1 l.) to the liquors then gave the sulphonium compound which formed large colourless plates from aqueous acetone, m.p. 134 °C (d.). (Found: C, 32·7; H, 5·6; N, 5·7; S, 12·0; Br, 30·5. Calc. for $C_7H_{14}O_2NS$ Br: C, 32·8; H, 5·5; N, 5·5; S, 12·5; Br, 31·2%.)

Reactions of the S-β-L-alanyltetrahydrothiophenium ion

(a) *With alkali.* The ion was very unstable above about pH 9 rapidly decomposing at room temperature to tetrahydrothiophene (80–90 per cent), characterized as its mercurichloride,[10] needles from methanol, m.p. 129–130 °C (lit. m.p. 129–130 °C), and its methiodide, m.p. 185–188 °C (lit. m.p. 185–190 °C[11]), not depressed on admixture with authentic samples.

Paper chromatography of the aqueous solution indicated the presence of a mixture of unidentified amino acids. However, when the solution was neutralized and electrolysed, L-alanine was detected on paper chromatograms. This was characterized by preparing its phenyluramino derivative by the general method of Loring and du Vigneaud.[9] It formed small plates from ethanol, chloroform and petroleum (b.p. 60–80 °C), m.p. 160 °C (d.), undepressed on admixture with an authentic sample. (Found: C, 59·2; H, 5·4; N, 13·3. Calc. for $C_{10}H_{12}O_3N_2$: C, 57·7; H, 5·8; N, 13·4%.)

(b) *Stability in water and acid.* The compound appeared to be relatively stable in hot water and at lower pH values.

(c) *Thermal decomposition.*[12] S-β-L-Alanyltetrahydrothiophenium bromide (3 g) was heated in an oil bath at (140–160 °C) when immediate decomposition occurred yielding an oil (0·9 g) which was largely tetrahydrothiophene as shown by preparation of its mercurichloride.

(d) *Electrolysis.* S-β-L-Alanyltetrahydrothiophenium bromide (1 g) in water (10 ml) was electrolysed using a current of 0·5–0·8 A in a Shandon Desalter. The tetrahydrothiophene which was liberated was extracted into ether and estimated as its mercurichloride (yield = 80–90 per cent). The aqueous phase was found to contain only L-alanine (0·45 g) which was characterized as its phenyluramino derivative as previously described. (Found: C, 57·2; H, 5·4. Calc. for $C_{10}H_{12}O_3N_2$: C, 57·7; H, 5·8%.)

(e) *Reaction with L-cysteine at pH 7.* S-β-L-Alanyltetrahydrothiophenium bromide (0·51 g) was dissolved in water (2 ml) and treated with a solution of L-cysteine (0·24 g) in 4 N sodium hydroxide (0·5 ml). The pH of the solution was adjusted to 7 if necessary with dilute acid, and from then on the pH of the mixture was frequently tested and never allowed to exceed 7. After heating on the steam bath for 3 hr the mixture, which contained a small quantity of solid, was extracted with ether. After drying and evaporating the ether the residue was treated with a concentrated solution of mercuric chloride in methanol, and the resulting solid (20 mg) was found to be tetrahydrothiophene mercurichloride. The solid (0·09 g) which separated from the original reaction mixture was shown by paper chromatography to consist of cysteine and S-dicysteinylbutane.

A similar result was obtained when the reaction was carried out in 50 per cent acetone.

(f) *Reaction with cysteine at pH 8–9.* S-β-L-Alanyltetrahydrothiophenium bromide (2·56 g) was dissolved in water (5 ml) and treated with a solution of L-cysteine (1·21 g) in 4 N NaOH (2·5 ml). After heating at 100 °C for a few minutes a copious solid separated. After 0·5 hr the mixture was cooled, treated with methanol (5 ml), and the solid was separated by filtration and washed with methanol. The solid (1·8 g) formed a phenyluramino derivative, plates from a mixture of chloroform, ethanol, and petrol (b.p. 60–80 °C), m.p. 158 °C, undepressed on admixture with a sample of bis-phenyluraminodi-L-cysteinylbutane prepared from S-di-L-cysteinylbutane obtained by reacting Myleran directly with L-cysteine. The filtrate was extracted with ether, and after washing with water and drying (Na_2SO_4) the ether was evaporated. The residue yielded tetrahydrothiophene mercurichloride (0·1 g) on treatment with mercuric chloride in methanol.

The filtrate from the original reaction mixture was found to contain mainly cystine, while no lanthionine could be detected on paper chromatograms.

(g) *Reaction with cysteine in the presence of aqueous sodium hydroxide.* S-β-L-Alanyltetrahydrothiophenium bromide (0·75 g) was dissolved in water (3 ml) and treated with a solution of L-cysteine (0·12 g) in 4 N NaOH (0·75 ml), giving a solution of pH > 10. After heating on the steam bath for 0·5 hr, the oil which had separated was extracted with ether. After drying (Na_2SO_4) and evaporating the ether, the residue was treated with a concentrated solution of mercuric chloride in methanol. The tetrahydrothiophene mercurichloride (0·84 g) corresponding to 0·21 g tetrahydrothiophene (approx. 80 per cent based on sulphonium compound), had m.p. 128 °C, undepressed on admixture with an authentic sample. The aqueous phase was neutralized with dilute hydrochloric acid and a solid (0·13 g) separated. Chromatography and co-chromatography in three solvent systems showed the mixture to consist of S-dicysteinylbutane and cystine, while no lanthionine was detected.

Paper chromatograms of the remaining aqueous solution indicated that no sulphonium compound remained unchanged, while no other identifiable ninhydrin-positive

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compounds were present. When the solution was electrolysed, and chromatographed, alanine was detected, and characterized as its phenyluramino derivative as described earlier.

The run proceeded similarly in 50 per cent acetone, and at room temperature if the mixture was allowed to stand for a longer period.

Electrolysis of a mixture of pyruvic acid and ammonia in water

Pyruvic acid (0·5 g) was treated with an excess of 4 N ammonia and the mixture electrolysed for 0·5 hr. Alanine was the only ninhydrin-positive compound which was produced. Evaporation yielded alanine (0·48 g) characterized as its phenyluramino derivative.

Reaction of dimethanesulphonyloxypropane with L-*cysteine*

1:3-Dimethanesulphonyloxybutane (4·64 g) was dissolved in 50 per cent aqueous ethanol (150 ml) and treated with a mixture of L-cysteine hydrochloride (6·28 g, 2 Eq.) and 4 N caustic soda (20 ml, 4 Eq.) in ethanol (20 ml). After standing for 24 hr at room temperature, the precipitated solid was filtered off. Paper chromatography indicated the presence of a new ninhydrin-positive compound which was characterized as S-di-L-cysteinylpropane by preparation of its bisphenyluramino derivative. This formed small prisms from ethanol. m.p. = 176 °C. (Found: C, 53·1; H, 5·4; N, 10·8. Calc. for $C_{23}H_{28}O_6N_4S_2$: C, 53·6; H, 5·4; N, 11·1%.)

Reaction between Myleran and mercaptoethylamine

Preparation of aminoethyltetrahydrothiophenium mesylate. A solution of mercaptoethylamine hydrochloride (0·46 g) in water (4 ml) and 2 N NaOH (4 ml) was added to a hot solution of Myleran (0·96 g) in acetone (20 ml). The clear solution was warmed for 2 hr on a steam bath and then evaporated to dryness under reduced pressure. Dilution of an ethanolic extract of the residue with dry ether gave an oil, which formed aminoethyltetrahydrothiophenium dipicrate, m.p. 203 °C, with sodium picrate. (Found: C, 36·7; H, 3·2; N, 17·0; S, 5·4. Calc. for $C_{18}H_{19}O_{14}N_7S$: C, 37·0; H, 3·5; N, 17·5; S, 5·1%.)

Paper chromatography of a solution of the original oil showed the presence of a ninhydrin-positive compound which disappeared, giving rise to a new spot when the solution was electrolysed. When the sulphonium picrate was converted to the chloride and run on a similar two-way chromatogram only one compound was present which corresponded in R_f value to the original compound.

The reaction of Myleran with glutathione

(a) *In water.* Myleran (0·62 g) was dissolved in hot water (10 ml) and treated with a solution of glutathione (0·76 g) in 4 N sodium hydroxide (2·5 ml). The mixture was heated on a steam bath for 1·75 hr. The cooled solution was extracted with ether and the tetrahydrothiophene estimated as its mercurichloride. The yield was usually about 35 per cent. Paper chromatography of the aqueous layer indicated that some breakdown of the peptide had occurred. Hydrolysis with an equal volume of concentrated hydrochloric acid yielded glycine, glutamic acid, lanthionine, and a small quantity of cystine.

Lanthione was also produced at shorter reaction times, but if a greater excess of alkali was used the yield of lanthionine was greatly reduced.

Lanthionine was not isolated from the reaction mixture and so its concentration could not be estimated. Traces of lanthionine were sometimes produced when glutathione (0·76 g) was heated with 4 N NaOH (2·5 ml) in the absence of Myleran and then hydrolysed.

(b) *In aqueous acetone.* Myleran (62 mg) was dissolved in acetone (3 ml) and treated with a solution of glutathione (153 mg) in a mixture of 1 N NaOH (1·5 ml) and water (2 ml). After standing overnight the tetrahydrothiophene which had separated was extracted with ether and estimated as its mercurichloride (Yield $< 50\%$).

Paper chromatography of the aqueous layer indicated that some breakdown of the peptide had occurred. The aqueous layer was hydrolysed by refluxing with an equal volume of concentrated hydrochloric acid for 6 hr. Chromatography and co-chromatography indicated that glutamic acid, glycine, and lanthionine were virtually the only ninhydrin-positive products present.

Identical runs carried out in the absence of Myleran indicated that lanthionine was not produced under these conditions.

The effect of alkali on cystine

A solution of cystine (240 mg) in 4 N NaOH (0·5 ml) was heated at 100 °C for 2 hr. Aliquots taken at intervals were neutralized and chromatographed. No lanthionine was formed, the cystine remaining apparently unchanged.

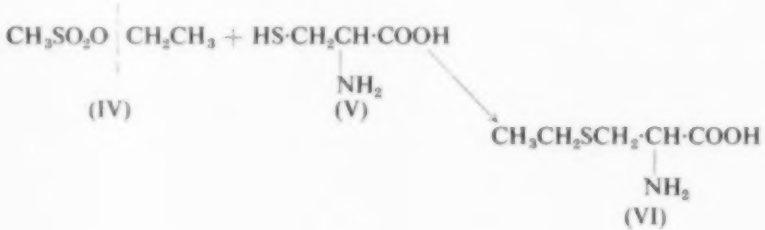
Reaction of Myleran with proteins

(a) *Wool.* Washed wool (25 g) reduced with calcium thioglycollate was suspended in water (200 ml) and 2 N NaOH (20 ml) and treated with a hot solution of Myleran (5 g) in water (300 ml). Steam distillation of the mixture yielded tetrahydrothiophene (in the initial runnings) which was characterized as its mercurichloride, m.p. 128–129 °C. Yield = 2·8 g (40 per cent of theoretical assuming that approximately 10 per cent of the reduced wool is cysteine).

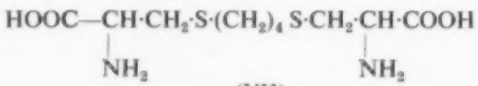
(b) *Egg albumen.* Myleran (5 g) in water (300 ml) was added to a solution of egg albumen (100 g) in water (200 ml) and 2 N NaOH (50 ml). The resulting mixture was steam distilled and tetrahydrothiophene appeared in the first runnings. Yield 120 mg, m.p. of mercurichloride 128–129 °C.

RESULTS AND DISCUSSION

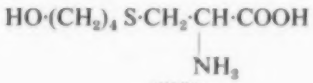
Ethyl methanesulphonate (IV) reacted readily with L-cysteine (V) in a slightly alkaline medium forming S-ethyl-L-cysteine (VI).[5]



It seemed likely therefore that Myleran (I) would react with L-cysteine in an analogous manner yielding S-di-L-cysteinylbutane (VII), if both alkylating centres reacted with cysteine, or S-(4-hydroxybutyl)-L-cysteine (VIII), if one alkylating centre reacted with cysteine while the other was hydrolysed.

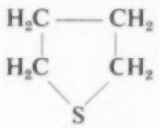


(VII)



(VIII)

However, when the reaction was carried out in hot aqueous solution at pH 8 using either 1 or 2 moles of cysteine, only relatively small quantities of these products were formed, the major product being a water-soluble amino acid which was isolated by the addition of a large quantity of acetone to the reaction mixture. While the product isolated by this method was contaminated with considerable quantities of salt, examination of its chemical properties was not precluded. In attempting to desalt the amino acid by electrolysing its aqueous solution, decomposition occurred giving rise to a strong-smelling water-insoluble oil identified as tetrahydrothiophene (IX):

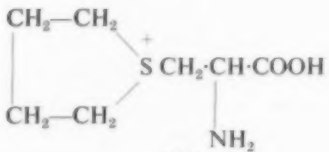


(IX)

Paper chromatograms of the aqueous phase showed alanine to be the only ninhydrin-positive product present, and this was later isolated and characterized as its phenyluramino derivative.

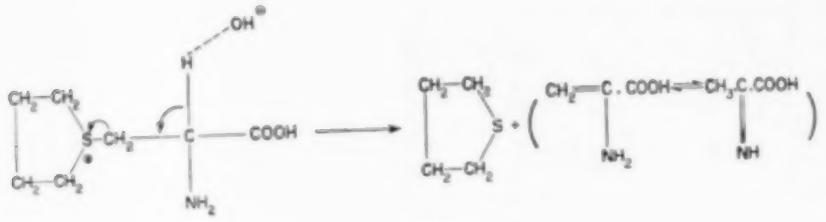
While the compound appeared to be relatively stable in aqueous solution, treatment with aqueous alkali again led to rapid decomposition to tetrahydrothiophene and some ninhydrin-positive material which on electrolysis in water was converted to alanine. Pyrolysis at 140 °C again yielded tetrahydrothiophene. The extreme water solubility of the product and the fact that it formed an immediate precipitate with sodium picrate (unlike alkylated cysteine) suggested the presence of a positively charged ion.

These data were consistent with the major product from the reaction of Myleran with L-cysteine being a sulphonium compound derived from the S-β-alanyltetrahydrothiophenium ion (X):



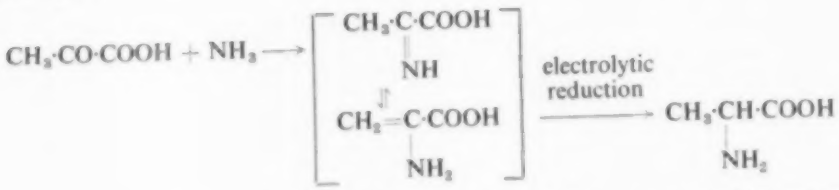
(X)

The formation of tetrahydrothiophene by the action of alkali probably occurred by the normal β-elimination of a proton



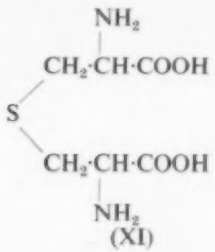
(XI)

The other product formed in this reaction was almost certainly aminoacrylic acid (in tautomeric equilibrium with iminopyruvic acid). This would account for the formation of alanine on electrolysis of the aqueous phase of the reaction mixture, since a mixture of pyruvic acid and ammonia was also converted to alanine on electrolysis.



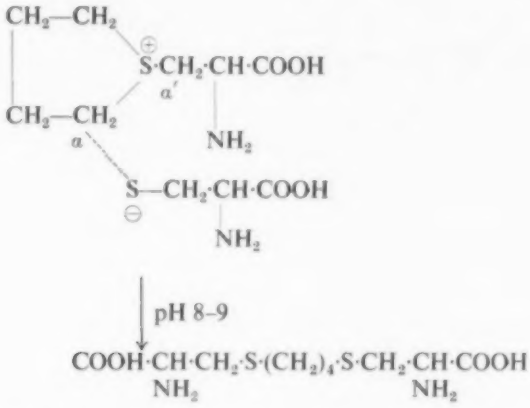
The sulphonium compound could not be freed from salt, but treatment of its aqueous solution with sodium picrate yielded a picrate, m.p. 135 °C, which was not depressed on admixture with the picrate prepared from S-β-alanyltetrahydrothiophenium chloride, made unambiguously by heating S-(4-hydroxybutyl)-L-cysteine (VIII) with hydrochloric acid. When 1:4-dibromobutane was reacted with L-cysteine in aqueous acetone a similar reaction occurred to that with Myleran, yielding S-β-alanyltetrahydrothiophenium bromide m.p. 134 °C (d.), and since it was easily isolated in a pure state and underwent similar chemical reactions to the Myleran product, it was used in many of the *in vitro* studies.

Since it was considered that this or a related sulphonium ion might be formed *in vivo* during the metabolism of Myleran, it was relevant to determine whether the sulphonium compound was itself an alkylating agent. As previously reported[7] this sulphonium compound does not react with water or cysteine at pH 7 or produce S-(4-hydroxybutyl)-cysteine or S-dicysteinylbutane *in vivo*. It has since been established that it does react with nucleophilic reagents under appropriate experimental conditions, the pH of the medium being the major controlling factor. S-β-L-alanyltetrahydrothiophenium bromide was relatively stable at pH 7 and when treated with cysteine under these conditions, either in water or in aqueous acetone, almost no reaction occurred, probably due to there being virtually no ionized cysteine at this pH. However, when the pH of the medium was raised to 8–9 a rapid reaction occurred even at room temperature, and S-dicysteinylbutane separated in high yield. An examination of the reaction mixture indicated the absence of lanthionine (XI) which would have



(XI)

been produced had the ionized cysteine attacked the α'-carbon atom instead of one of the α-carbon atoms.



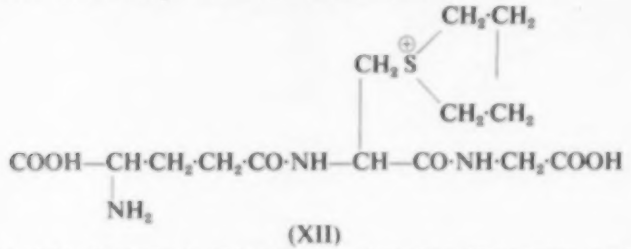
There was virtually no tetrahydrothiophene liberated under these conditions, while formation of lanthionine would have involved its simultaneous liberation in equivalent amount. The method is suggested as very satisfactory for making S-dicysteinylbutane.

In the presence of an excess of strong alkali very little reaction occurred between L-cysteine and the sulphonium compound, as judged by the liberation of an 80 per cent yield of tetrahydrothiophene together with only a small quantity of S-di-L-cysteinylbutane. The tetrahydrothiophene was produced by hydroxyl ion attack on the β-hydrogen atom as discussed earlier, rather than by attack of the ionized cysteine on the α'-carbon atom (see above scheme) since no lanthionine was formed. Paper chromatography of the remaining aqueous phase showed that no naturally occurring amino acids were present. However, electrolysis produced L-alanine indicating the presence of the pyruvic acid–ammonia tautomeric mixture, and supporting the postulated mechanism for the formation of tetrahydrothiophene.

The reaction of Myleran with glutathione

The course of the reaction between Myleran and glutathione was studied since the latter is present in high concentration in the mammal, and moreover it provides an example of a compound containing "bound" cysteine. Since the pK of the thiol group of glutathione is higher than that of cysteine, it was not surprising that the rate of reaction was relatively slow until the pH of the solution was made greater than 8. A sulphonium compound of structure (XII) was almost certainly formed, but its

isolation was precluded since subsequent rapid decomposition to tetrahydrothiophene also occurred at the pH of the reaction mixture.

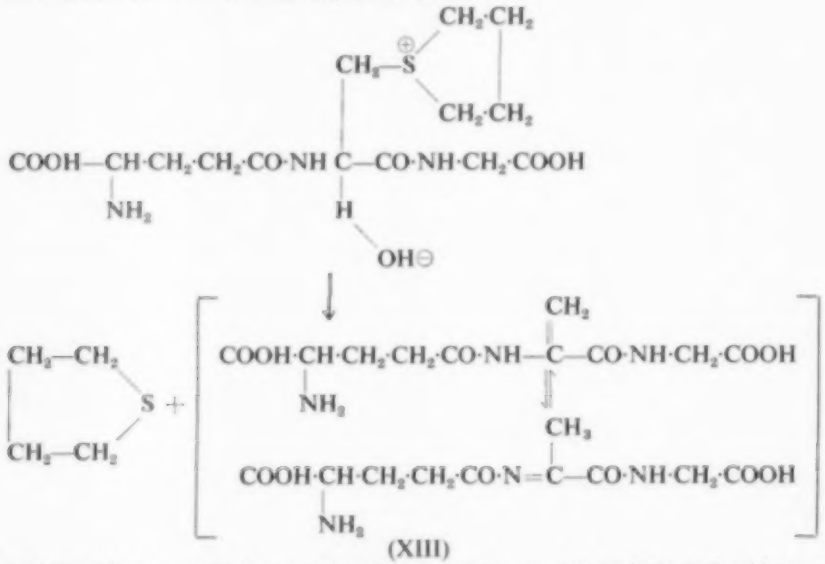


(XII)

However, the yield of tetrahydrothiophene was less than 50 per cent, probably partly due to alkaline hydrolysis of some of the Myleran.

Paper chromatography of the aqueous solution revealed that some decomposition of the peptide had occurred during the Myleran treatment. After hydrolysis with hydrochloric acid paper chromatography revealed that glycine, glutamic acid and lanthionine were the only major ninhydrin-positive compounds present. There was a striking absence of S-(4-hydroxybutyl)-L-cysteine and S-di-L-cysteinylbutane in the hydrolysed reaction mixture.

Two possible mechanisms are suggested for the formation of lanthionine, although it has not been possible to determine which was operative. If the sulphonium compound was attacked by alkali in a manner analagous to the S-β-L-alanyltetrahydrothiophenium ion then tetrahydrothiophene would be liberated with the simultaneous formation of a tautomeric dehydropeptide (XIII).

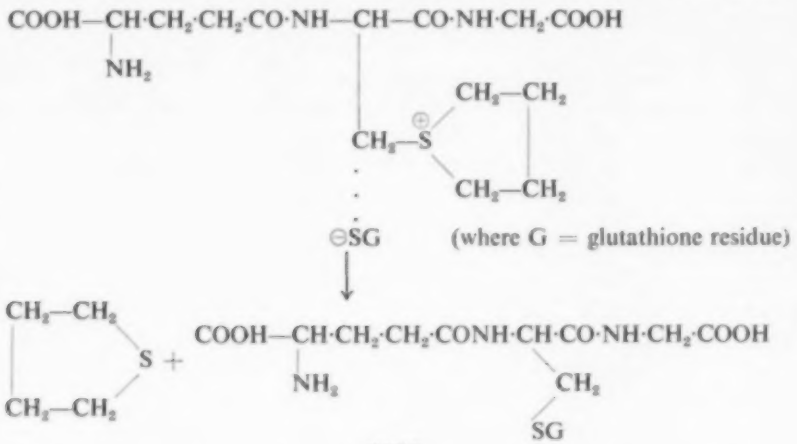


(XIII)

Addition of an unreacted molecule of glutathione across the ethylenic double bond would yield bound lanthionine (XIV) from which lanthionine would be liberated on hydrolysis. Greenstein[13] has demonstrated that in general dehydropeptides readily undergo addition reactions of this type. Birch[14] considers that dehydropeptides are

relatively unstable and this might account for the presence of decomposition products in the Myleran–glutathione reaction mixture prior to acid hydrolysis.

The other possible mechanism for the production of lanthionine would involve attack of the sulphonium compound by ionized glutathione as indicated below.

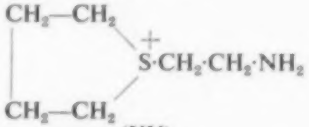


(XIV)

In this case attack of the carbon atom indicated rather than the ring carbon atom such as was observed when L-cysteine reacted with the S-β-L-alanyltetrahydrothiophenium ion, would probably be favoured because of the electron-attracting substituents attached to the neighbouring carboxyl and amino groups. Either mechanism would account for the low yield of tetrahydrothiophene formed.

These studies provide an interesting comparison between the reactivities of two sulphydryl compounds, cysteine and glutathione, towards Myleran. Sulphonium compounds were readily formed, and both yielded tetrahydrothiophene under appropriate conditions. The ease of formation of S-di-L-cysteinylbutane by reaction of the S-β-alanyltetrahydrothiophenium ion with L-cysteine would suggest that if 2:3-^{14}C-Myleran reacted with free L-cysteine *in vivo* then this compound might be formed as a radioactive metabolite. However, the dicysteinyl compound would probably not be formed if reaction occurred *in vivo* with glutathione, or presumably with higher peptides or proteins containing fully bound cysteine.

The thiol proteins kerateine and egg albumen both yielded tetrahydrothiophene on treatment with Myleran in the presence of alkali, indicating the general nature of this reaction with cysteine-containing compounds. The more general nature of the reaction was established by showing that a sulphonium compound (XV) was produced when Myleran was reacted with β-mercaptoethylamine,



(XV)

and that tetrahydrothiophene was liberated when thioglycolic acid was treated with Myleran in the presence of alkali.

On the basis of these results it seemed likely that if 2:3-^{14}C-Myleran reacted with the thiol group of cysteine or cysteine-containing compounds *in vivo* then the radioactive urinary metabolites would probably be derived from S-dicysteinylbutane (VII), S-(4-hydroxybutyl)-cysteine (VIII) or the major product of reaction, the S-β-alanyltetrahydrothiophenium ion (X). Secondary changes could also result in the replacement of cysteinyl residues by lanthionine or other amino acids.

SUMMARY

(1) The reaction between Myleran and L-cysteine yielded mainly S-β-L-alanyltetrahydrothiophenium mesylate. S-di-L-cysteinylbutane was also produced in low yield.

(2) Dibromobutane reacted in an analagous manner with L-cysteine yielding S-β-L-alanyltetrahydrothiophenium bromide and S-di-L-cysteinylbutane.

(3) Myleran reacted with glutathione at pH 8–9 to form tetrahydrothiophene and bound lanthionine.

(4) Myleran reacted with both kerateine and egg albumin at pH $>$ 10, liberating tetrahydrothiophene.

(5) Myleran reacted with β-mercaptoethylamine to form β-aminoethyltetrahydrothiophenium mesylate, and with thioglycolic acid in alkali to form tetrahydrothiophene.

(6) Treatment of the S-β-L-alanyltetrahydrothiophenium ion with aqueous alkali caused decomposition to tetrahydrothiophene and aminoacrylic acid.

(7) Thermal decomposition of S-β-L-alanyltetrahydrothiophenium bromide yielded mainly tetrahydrothiophene.

(8) Electrolysis of the S-β-L-alanyltetrahydrothiophenium ion yielded tetrahydrothiophene and L-alanine.

(9) No reaction occurred between the S-β-L-alanyltetrahydrothiophenium ion and L-cysteine at pH 7. At pH 8 a high yield of S-di-L-cysteinylbutane was produced, while at pH $>$ 10 a high yield of tetrahydrothiophene was formed together with some S-di-L-cysteinylbutane.

(10) Aminoacrylic acid was converted quantitatively to alanine on electrolysis.

Aknowledgements—We wish to thank Professor A. Haddow and Dr. L. A. Elson for their interest in the work. We are also grateful to Miss M. Moreton and Miss R. Cotter for technical assistance.

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THE MODE OF ACTION OF ALKYLATING AGENTS—III

THE FORMATION OF 3-HYDROXYTETRAHYDROTHIOPHENE-1:1-DIOXIDE FROM 1:4-DIMETHANESULPHONYLOXYBUTANE (MYLERAN), S-β-L-ALANYLTETRAHYDROTHIOPHENIUM MESYLATE, TETRAHYDROTHIOPHENE AND TETRAHYDROTHIOPHENE-1:1-DIOXIDE IN THE RAT, RABBIT AND MOUSE

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(*Received* 6 *September* 1960)

Abstract—The metabolism of Myleran has been investigated in an attempt to explain the mechanism of its biological action. After injection of Myleran into the rat, rabbit and mouse at least 60 per cent of the urinary radioactivity is in the form of 3-hydroxytetrahydrothiophene-1,1-dioxide. S-β-L-alanyltetrahydrothiophenium mesylate, tetrahydrothiophene, and tetrahydrothiophene-1,1-dioxide are also metabolised to this compound. It is concluded that the major reaction which Myleran undergoes *in vivo* is reaction with cysteine or a cysteinyl moiety to form a cyclic sulphonium ion which undergoes decomposition to tetrahydrothiophene. This in turn is converted to tetrahydrothiophene-1,1-dioxide and thence to 3-hydroxytetrahydrothiophene-1,1-dioxide.

INTRODUCTION

EARLIER studies of the metabolism and distribution of Myleran by Peng[1] using the ^{35}S-labelled compound, and by Trams *et al.*[2] using 1:4-^{14}C-Myleran, have revealed the presence of new radioactive compounds in the urine of the human and the rat in addition to a small quantity of unchanged drug. Thus ^{35}S-methanesulphonic acid, the product which would inevitably be formed if Myleran alkylated nucleophilic centres *in vivo*, has been identified, and three ^{14}C-metabolites accounting for 70–80 per cent of the urinary radioactivity have been observed. This paper describes the chemical nature and the mode of formation of the major urinary metabolite.

MATERIALS AND METHODS

2:3-^{14}C-Myleran, specific activity 4 μc/mg was kindly supplied by Dr. P. Brookes of this Institute.

^{35}S-*Tetrahydrothiophene*

^{35}S-sodium sulphide (16 mg, specific activity 26 mc/mM) was dissolved in water and added to an aqueous solution of $Na_2S{\cdot}9H_2O$ (6·6 g) to give a total volume of 50 ml. This was added to a solution of 1:4-dibromobutane (5·4 g) in ethanol (50 ml), and the solution was made up to 180 ml with 50 per cent ethanol. The mixture was heated under reflux for 20 hr, and distilled until the distillate failed to give a precipitate with mercuric chloride. A concentrated solution of mercuric chloride in methanol was then added to the total distillate, and after cooling in ice the precipitated mercurichloride was separated by filtration. A rapid current of steam was

passed through an aqueous suspension of the mercurichloride until all the liberated ^{35}S-tetrahydrothiophene had distilled over. Isolation by ether extraction yielded 1·88 g of product.

Tetrahydrothiophene-1:1-*dioxide*

Redistilled tetrahydrothiophene (8·8 g)was treated with cooling with 30 per cent hydrogen peroxide (30 ml) and after the initial exothermic reaction had subsided the solution was refluxed for 1 hr. The sulphone isolated by continuous extraction with chloroform was distilled under reduced pressure yielding a solid, m.p. $>$ 20 °C (lit. m.p. = 28·86 °C [3]).

^{35}S-*Tetrahydrothiophene*-1:1-*dioxide*

This was synthesized in nearly quantitative yield by the above method using ^{35}S-tetrahydrothiophene (0·5 g) and 30 per cent hydrogen peroxide (1·8 ml).

^{35}S-*Tetrahydrothiophene methiodide*

This was synthesized using ^{35}S-tetrahydrothiophene. Needles from acetone, m.p. 185–190 °C (lit. m.p. = 185–190 °C).[4]

3-*Hydroxytetrahydrothiophene*-1:1-*dioxide dinitrobenzoate*

3-Hydroxytetrahydrothiophene-1:1-dioxide (Sulpholanol) (1 g) kindly obtained by Professor E. Boyland of this Institute, dinitrobenzoyl chloride (3 g) and pyridine (5 ml) were heated together at 100 °C for 0·5 hr. The reaction mixture was treated with 2 N sodium carbonate (100 ml) and the dinitrobenzoate was extracted into chloroform. The chloroform layer was washed successively with 2 N hydrochloric acid, and water, and then dried (sodium sulphate). The residual oil soon solidified and crystallized from methanol in almost colourless prisms, m.p. 195–197 °C. (Found: C, 40·3; H, 3·2; N,8·3; S, 9·5, mol.wt. 340. Calc. for $C_{11}H_{10}O_8N_2S$: C, 40·0; H, 3·0; N, 8·5; S, 9·7%; mol.wt. 330.)

Animals

Six-week-old male Wistar rats (approx. 200 g) and 3-month-old doe rabbits were used in the animal experiments.

Collection and assay of radioactive metabolites

The methods used to collect and assay the radioactive urine, faeces and exhaled carbon dioxide have been described.[5] The techniques used to prepare the urinary samples for chromatography were also as described for the study of the metabolism of ethyl methanesulphonate[6] ("half-Myleran").

All fractions were routinely run in the following three solvents: butanol–ethanol–propionic acid–water (20:10:10:4), solvent I; butanol–acetone–di*cyclo*hexylamine–water (20:20:10:4), solvent II; methylethylketone–acetic acid–water (3:1:1), solvent III.

RESULTS

The metabolism of Myleran

1. *Rat.* 2:3-^{14}C-Myleran (20 mg/kg in propylene glycol (2 ml) or arachis oil (2 ml)) was injected intraperitoneally into a male Wistar rat and the urine, faeces and exhaled carbon dioxide were collected and the radioactivity assayed (Table 1).

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Autoradiographs of paper chromatograms prepared from urine excreted during the first 24 hr after injection (Fig. 1A) showed that several radioactive metabolites were present, one of these constituting the major fraction (spot *a*, Fig. 1A). It has been shown that at least 60 per cent of the urinary radioactivity was associated with this metabolite by scanning the paper chromatograms with an end window counter. Autoradiographs of paper chromatograms prepared from the second-day's urine again confirmed the presence of the major metabolite in approximately the same relative proportion.

TABLE 1. AVERAGE PERCENTAGE OF INJECTED RADIOACTIVITY EXCRETED FROM THREE MALE WISTAR RATS AFTER INJECTION OF 2:3-^{14}C-MYLERAN

Times after i.p. injection (hr)	Urine (%)	Faeces	CO_2 (%)
6–24	28–35	Negligible	4–8
24–48	12–15	Negligible	Negligible
48–72	Negligible	Negligible	Negligible

The polar nature of the major metabolite was emphasized by the need for prolonged continuous chloroform extraction to isolate it from the aqueous phase: characterization of the major metabolite was precluded at this stage because of the necessarily small quantities of Myleran which could be safely administered.

While the major metabolite has since been identified, it has not been possible to elucidate the nature of the other radioactive compounds present in the urine in much lower concentration.

2. *Rabbit and mouse.* Several rabbits were injected intraperitoneally with 2:3-^{14}C-Myleran (4 mg in arachis oil (2 ml)) and the urines examined separately. Autoradiographs of paper chromatograms again showed that the major proportion of the urinary radioactivity was associated with one metabolite which had chromatographic and chemical properties identical with that excreted by the rat. Subsequent isolation confirmed this identity.

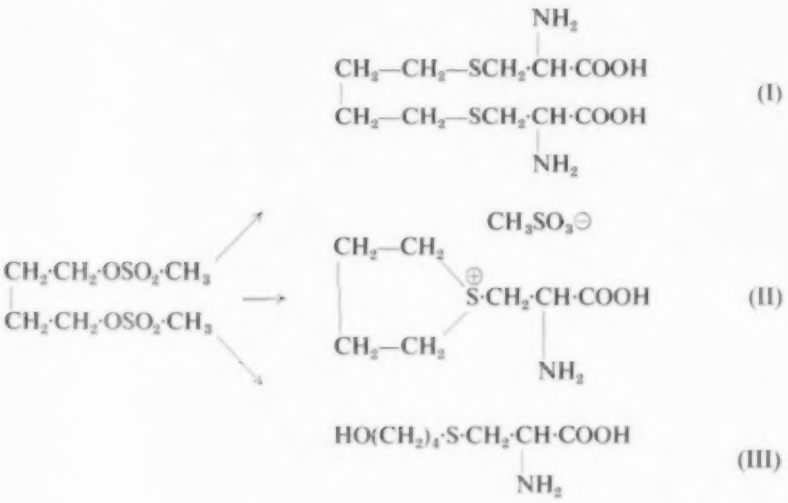
Twenty mice were injected intraperitoneally with 2:3-^{14}C-Myleran (0·6 mg in arachis oil (0·5 ml)) and the bulked urine examined as before. A large proportion of the major metabolite was excreted, but inspection of autoradiographs indicated that the other metabolites differed from those excreted by the rat and the rabbit.

The properties of the major radioactive urinary metabolite excreted after injection of 2:3-^{14}C-Myleran

On the basis of the observed formation of ethyl mercapturic acid from ethyl methanesulphonate (half-Myleran) *in vivo*,[5] it was considered that Myleran might undergo a similar conversion *in vivo* leading to the production and excretion of conjugates of sulphur-containing amino acids.

The formation of S-di-L-cysteinylbutane (I), S-β-L-alanyltetrahydrothiophenium mesylate (II), and S-(4-hydroxybutyl)-L-cysteine (III), by reaction of Myleran with L-cysteine, and the formation of sulphonium compounds from glutathione and proteins *in vitro* has been described earlier.[6]

However, the major metabolite differed in its R_f value from products (I), (II) and (III), and moreover did not contain an amino or carboxyl group as shown by its free passage through ion exchange columns, and its negative response to ninhydrin. Even in high concentration the metabolite did not react with the platinic iodide reagent and was stable to oxidizing agents, indicating the absence of a mercaptan or sulphide grouping, but not ruling out the presence of sulphur in a more oxidized state. The stability of the metabolite to hot concentrated hydrochloric acid showed that it was not a conjugate.



While, therefore, the known products of the reaction between Myleran and L-cysteine were not present as urinary metabolites of Myleran, the possibility of one of these being a precursor was not excluded. Accordingly, the major product of this reaction, S-β-L-alanyltetrahydrothiophenium mesylate (II), was prepared from 2:3-^{14}C-Myleran, and injected into rats to determine whether any of its urinary metabolites were identical to those excreted after Myleran injection. It was considered that if reaction occurred with a thiol-containing peptide or protein *in vivo* to form a sulphonium compound, the final radioactive urinary metabolites might nevertheless be identical to those formed via the cysteine reaction.

The metabolism of S-β-L-*alanyltetrahydrothiophenium mesylate* (II) *in the rat*

The exhaled carbon dioxide and faeces contained negligible quantities of radioactivity while 32 per cent of the injected radioactivity was excreted in the urine during the first 24 hr. Autoradiographs of paper chromatograms of the urine indicated the presence of one major metabolite (spot *a*, Fig. 1B), different from the injected sulphonium compound, but identical to the major Myleran metabolite in both R_f value (solvents I, II, III) and chemical properties. Again continuous extraction of the urine with chloroform separated the major metabolite. It was thus evident that sulphonium ion formation involving either cysteine or a cysteine-containing compound was probably the initial major reaction which Myleran underwent *in vivo*.

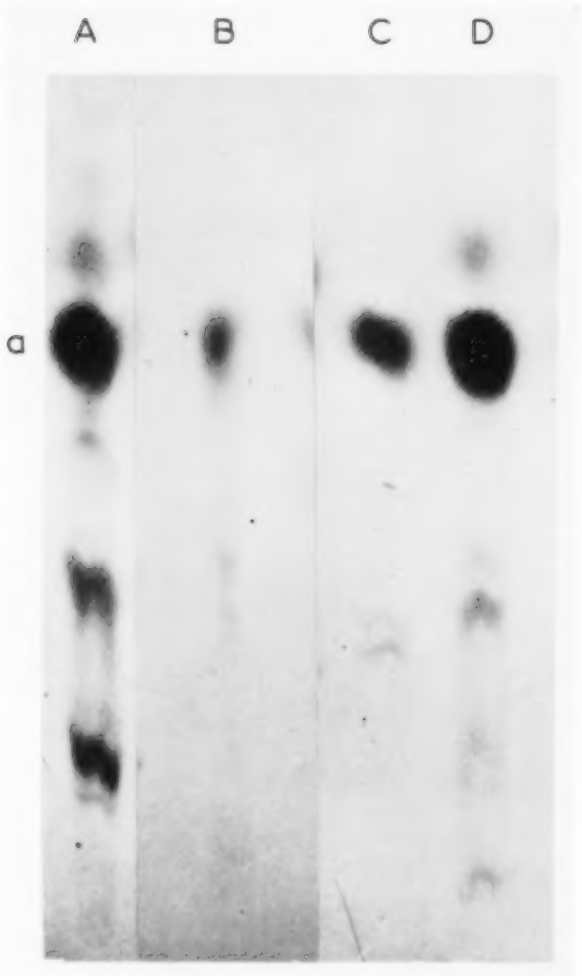


FIG. 1. Autoradiograph of a unidimensional paper chromatogram (solvent I) of urine excreted from the rat after injection of 2:3-^{14}C-Myleran (A), 2:3-^{14}C-S-β-alanyltetrahydrothiophenium mesylate (B), ^{35}S-tetrahydrothiophene (C), and ^{35}S-tetrahydrothiophene-1:1-dioxide (D). Spot *a* is radioactive 3-hydroxytetrahydrothiophene-1:1-dioxide.

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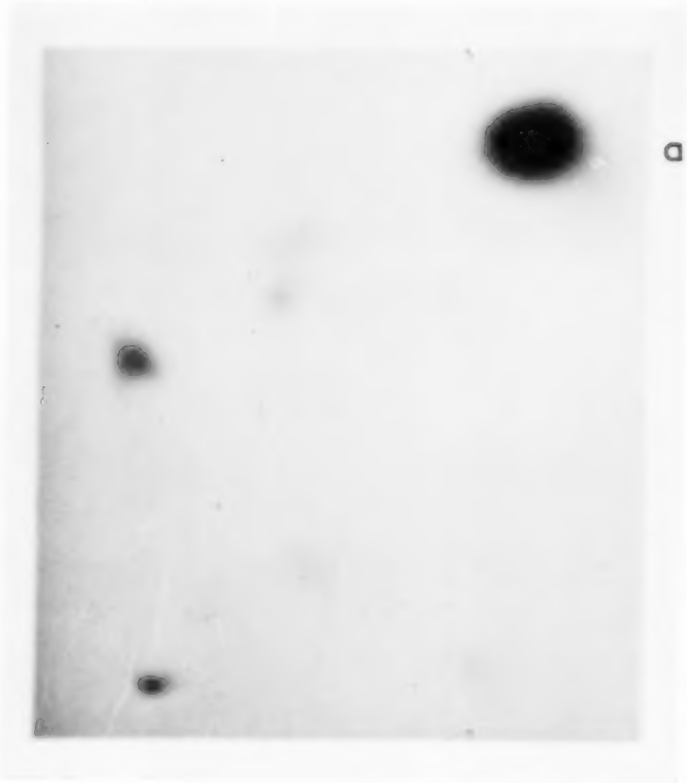
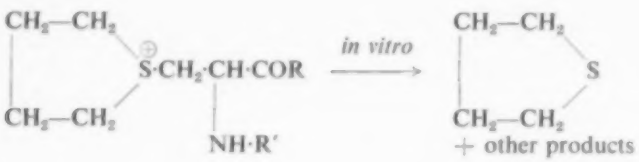


FIG. 2. Autoradiograph of a two-dimensional paper chromatogram (solvents I and II) showing the position and relative concentration of radioactive 3-hydroxytetrahydrothiophene-1:1-dioxide. (Spot *a*) excreted after injection of 2:3-^{14}C-Myleran. Solvent I →. Solvent II ↑.

The metabolism of ^{35}S-*tetrahydrothiophene in the rat*

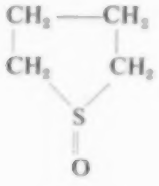
The ease of formation of tetrahydrothiophene (IV) under mild conditions from the S-β-L-alanyltetrahydrothiophenium cation *in vitro* and from the sulphonium compounds derived from the reaction of Myleran with glutathione and proteins *in vitro* led to a consideration of the possibility of its being formed also *in vivo*.



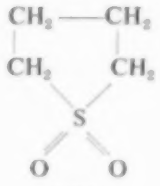
(IV)

(R = OH, peptide or protein residue)
(R′ = H, peptide or protein residue)

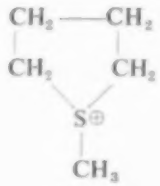
The chemical properties of the major metabolite excreted after injection of Myleran or S-β-L-alanyltetrahydrothiophenium mesylate differed from those of tetrahydrothiophene. It was possible, however, that tetrahydrothiophene was formed initially by the decomposition of the sulphonium compound, and then further metabolized. When ^{35}S-tetrahydrothiophene was injected intraperitoneally into the rat (10 mg in arachis oil (2 ml)) one major radioactive metabolite was present in the first-day's urine, (spot *a*, Fig. 1C) accounting for at least 85 per cent of the urinary radioactivity. This was shown to possess the same chromatographic and chemical properties as the major metabolite formed from either Myleran or S-β-L-alanyltetrahydrothiophenium mesylate (spot *a*, Fig. 1A and 1B). No unchanged tetrahydrothiophene was detected either in the urine or in the exhaled air. Since the major metabolite from ^{35}S-tetrahydrothiophene failed to give a positive test for a thioether, thiol or disulphide (platinic iodide reagent), but clearly contained sulphur, it was evident that the sulphur atom was in some way modified. Possible mechanisms included oxidation to form tetrahydrothiophene sulphoxide (V), tetrahydrothiophene-1:1-dioxide (VI), or *in vivo* methylation to form the S-methyltetrahydrothiophenium cation (VII) (by analogy with the *in vivo* methylation of pyridine).



(V)



(VI)



(VII)

However, ^{35}S-tetrahydrothiophenium methiodide had a different R_f value from the major metabolite, and moreover when injected into rats (5 mg in water (2 ml)) it was

apparently excreted unchanged, a finding consistent with the stability of the methiodide when heated with caustic soda solution (cf. S-β-L-alanyltetrahydrothiophenium mesylate and bromide).

The stability of the major metabolite to hot concentrated hydrchloric acid and to powerful oxidizing agents proved that the compound was not a sulphoxide, and strongly favoured its being a sulphone; although it was not tetrahydrothiophene-1:1-dioxide the possibility of its being a derivative of this was next investigated.

The metabolism of ^{35}S-*tetrahydrothiophene*-1:1-*dioxide* (VI)

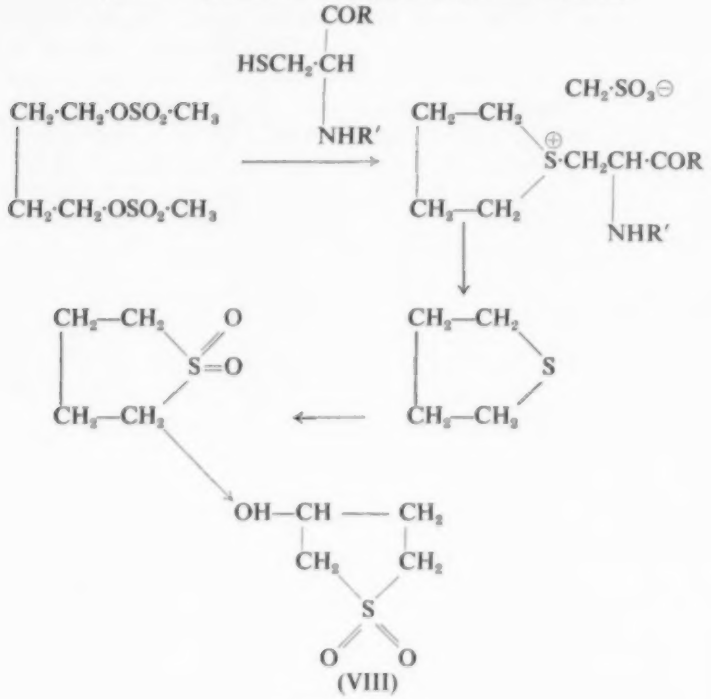
^{35}S-Tetrahydrothiophene-1:1-dioxide was injected intraperitoneally into three male Wistar rats (100 mg/rat in water (2 ml)) and the 24-hr urinary samples were examined separately. Again one major metabolite was excreted, constituting at least 85 per cent of the urinary radioactivity (spot *a*, Fig. 1D). It was shown to be identical in its chromatographic and chemical properties to the major metabolite excreted after injection of 2:3-^{14}C-Myleran, 2:3-^{14}C-S-β-L-alanyltetrahydrothiophenium mesylate, and ^{35}S-tetrahydrothiophene. The chemical properties of the major metabolite were not consistent with its being a further oxidation product such as a ketone, a diacid or a dehydro compound, and hence a remaining likely possibility was that ring hydroxylation had occurred, particularly in view of the enhancement of the reactivity of the ring carbon atoms towards nucleophilic reagents by the powerfully electron-attracting sulphone group. The presence of one or more hydroxyl groups in the metabolite was demonstrated by its reactivity towards acetyl chloride or benzoyl chloride in pyridine, leading to the formation of new products from which the metabolite could be regenerated by hydrolysis with mineral acid.

Tetrahydrothiophene-1:1-dioxide was found to be relatively non-toxic to the rat and the rabbit, and could thus be injected in sufficient quantity to enable isolation and characterization of the major metabolite. Three rabbits were each injected intraperitoneally with unlabelled tetrahydrothiophene-1:1-dioxide (1 g) and ^{35}S-tetrahydrothiophene-1:1-dioxide (100 mg), and after continuous extraction of the bulked urines with chloroform, the residual extract was treated with 3:5-dinitrobenzoyl chloride, yielding the dinitrobenzoate of 3-hydroxytetrahydrothiophene-1:1-dioxide (VIII). The ester formed pale yellow plates from methanol, m.p. 195–197 °C, undepressed on admixture with an authentic sample.

The same radioactive ester was prepared from the combined chloroform extracts of the urine obtained from two rabbits, one of which had received a small dose of 2:3-^{14}C-Myleran to act as carrier, and the other a relatively high dose of unlabelled tetrahydrothiophene-1:1-dioxide to enable isolation and characterization of the derivative. It was also obtained from the chloroform extract of urine from rats injected with high doses of S-β-L-alanyltetrahydrothiophenium mesylate (II) containing a radioactive tracer. Each derivative had m.p. 195–197 °C alone, and on admixture with an authentic sample. These latter experiments constituted proof that 3-hydroxytetrahydrothiophene-1:1-dioxide was produced *in vivo* from both Myleran and S-β-L-tetrahydrothiophenium mesylate.

It seems likely from the foregoing experiments that the major route of metabolism of Myleran *in vivo* is as follows:

(R = OH, amino acid, peptide or protein residue.)
(R′ = H, amino acid, peptide or protein residue.)



(VIII)

DISCUSSION

Urinary radioactivity

Since the urinary radioactivity was estimated by evaporating samples on plastic planchettes with an infra-red lamp, and in view of the steam volatility of both tetrahydrothiophene-1:1-dioxide and its 3-hydroxy derivative, the values recorded must represent a minimum value for the total radioactivity present.

In comparing the metabolic fate of ethyl methanesulphonate and Myleran it is of interest that while 30 per cent of the injected ^{14}C-ethylmethanesulphonate was converted to ^{14}C-carbon dioxide, only 4–7 per cent of the 2:3-^{14}C-Myleran was metabolized in this way. This result indicates that *in vivo* hydrolysis of Myleran was not a major metabolic pathway, since its hydrolysis product 1:4-(2:3-^{14}C)-butanediol was converted almost quantitatively to exhaled ^{14}C-carbon dioxide *in vivo*.

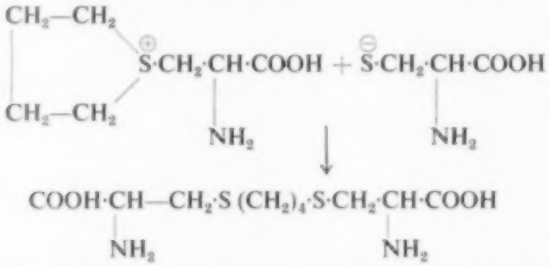
The metabolism of S-β-L-*alanyltetrahydrothiophenium mesylate*, (II), *tetrahydrothiophene*, (IV), *and tetrahydrothiophene*-1:1-*dioxide*, (VI)

Comparatively little is known of the nature of the intermediate products formed during the metabolism of sulphonium compounds in the mammal, since sulphate is the only urinary metabolite which has been reported following administration of certain sulphonium compounds to animals.[7] However, some sulphonium compounds are capable of survival since Challenger has reported the presence of sulphonium compounds in dog urine,[8] and S-methyltetrahydrothiophenium iodide, (VII), appears to be excreted unchanged after administration to the rat.

In the case of the S-β-L-alanyltetrahydrothiophenium ion, (II), it is certain that cleavage to tetrahydrothiophene, (IV), is the major, if not the exclusive, primary route of metabolism in the rat, rabbit and the mouse. An analogy can thus be drawn between the metabolism of this sulphonium compound and its hydrolytic decomposition with alkali (pH > 10). It will be recalled, however, that the sulphonium compound (II) was relatively stable in aqueous solution at physiological pH even at 100 °C, and this would strongly suggest that decomposition *in vivo* is controlled enzymatically. In this connexion the work of Cantoni and Anderson[9] is very relevant since they have isolated an enzyme from a marine alga which rapidly decomposes dimethylpropiothetin to dimethyl sulphide and acrylic acid, the same products as are formed by treatment with hot sodium hydroxide.

$$(CH_3)_2\overset{\oplus}{S}\cdot CH_2\cdot CH_2\cdot COOH \xrightarrow[\text{or hot NaOH}]{\text{enzyme, pH 5}} (CH_3)_2\cdot S + CH_2{=}CH\cdot COOH$$

It was shown in Part II[6] that the S-β-L-alanyltetrahydrothiophenium ion reacted rapidly and almost quantitatively with cysteine at about pH 8, yielding S-di-L-cysteinylbutane, and this reaction might be expected to occur *in vivo*.



However, metabolites of S-di-L-cysteinylbutane were not detectable in the urine from rats which had been injected with large doses of (II).

In general organic sulphides appear to be excreted with the sulphur atom intact, or else completely oxidized to sulphate.[10] The excretion of small quantities of a conjugate of S-ethyl-L-cysteine-1:1-dioxide after injection of ethyl methanesulphonate or S-ethylcysteine,[5] and the conversion of tetrahydrothiophene to 3-hydroxytetrahydrothiophene-1:1-dioxide, appear to be the first demonstrations of the conversion of sulphides to sulphones *in vivo*.[10]

Studies of the reactivity of tetrahydrothiophene-1:1-dioxide towards nucleophilic reagents have revealed that substitution normally occurs in the 2-position.[11] While steric effects are of secondary importance in these reactions, they will exert greater influence as the size of the attacking reagent increases, so that in the case of an enzyme-mediated reaction the less reactive, but less sterically-hindered 3-position obviously becomes the more vulnerable to attack.

In general phenols and alcohols are excreted as glucuronides and sulphates,[12] but 3-hydroxytetrahydrothiophene-1:1-dioxide was either not esterified *in vivo*, or formed an extremely labile ester which was not detectable by the methods employed.

The origin of the sulphur in 3-hydroxytetrahydrothiophene-1:1-dioxide

Since the reaction between Myleran and an ionized sulphydryl group to form a cyclic sulphonium compound appears to be general,[6] the sulphur atom in the Myleran

metabolite (VIII) could conceivably be derived from any naturally occurring sulphydryl compound such as coenzyme-A or homocysteine as well as from cysteine and cysteine-containing compounds. However, ethylmethanesulphonate (half-Myleran) reacted almost exclusively with the sulphydryl group of a cysteinyl moiety[5] and it would seem, therefore, by analogy that this is most probably the major source of the sulphur in the Myleran metabolite.

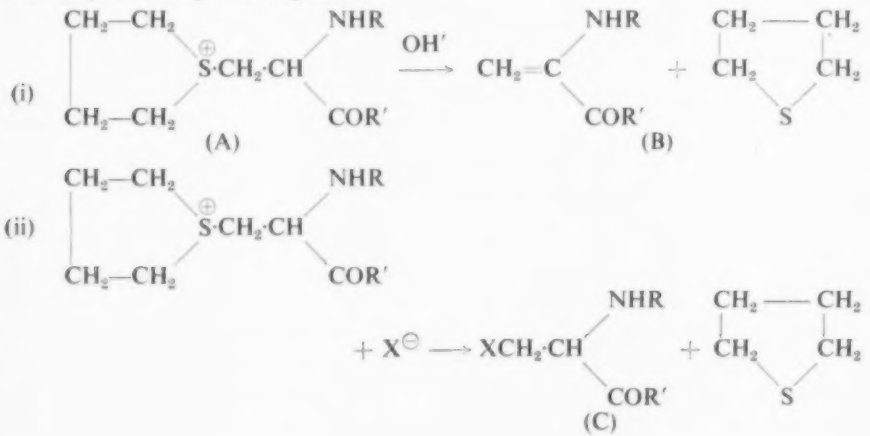
Possible relevance of these findings to the mode of action of Myleran

As stated earlier[5] the objective throughout the work described here and that described earlier on the metabolism of ethyl methanesulphonate[5] was to provide a comparison between the *in vivo* and *in vitro* reactions of two compounds, one possessing one alkylating centre and the other two, since compounds having two reactive centres separated by an optimum distance often manifest enhanced biological effects compared with their monofunctional analogues. For example Myleran, unlike ethylmethanesulphonate, exerts a profound effect on the bone marrow,[13] and has been shown to be a carcinogen in the rat.[14]

The study of the *in vivo* and *in vitro* reactions of Myleran with the thiol group has indicated three mechanisms by which the drug might modify the function of thiol-containing compounds in the cell. These include the initial sulphonium ion formation (A) which occurs *in vivo*, and which is obviously dependent on the bifunctionality of the drug, and the number of atoms in the chain separating the alkylating centres. Rings of greatest stability will be formed from diesters with a chain length of four or five carbon atoms. In this connexion it has been observed that maximum biological activity in the series of bismethanesulphonyloxy esters is associated with these particular chain lengths. Sulphonium ion formation of this type involves not only alkylation of the thiol group, which can also be accomplished by the monofunctional alkylating agents *in vivo*,[5] but also an alteration of the electrical properties of the molecule.

Further modification of the original amino acid, peptide, or protein would result from the subsequent formation of tetrahydrothiophene, a reaction occurring *in vivo*, and involving removal of the sulphur atom from the amino acid moiety.

In vitro studies have indicated that the tetrahydrothiophene formed from sulphonium compounds can be produced either by hydrolytic cleavage (i), or by direct attack by a nucleophilic reagent (ii).

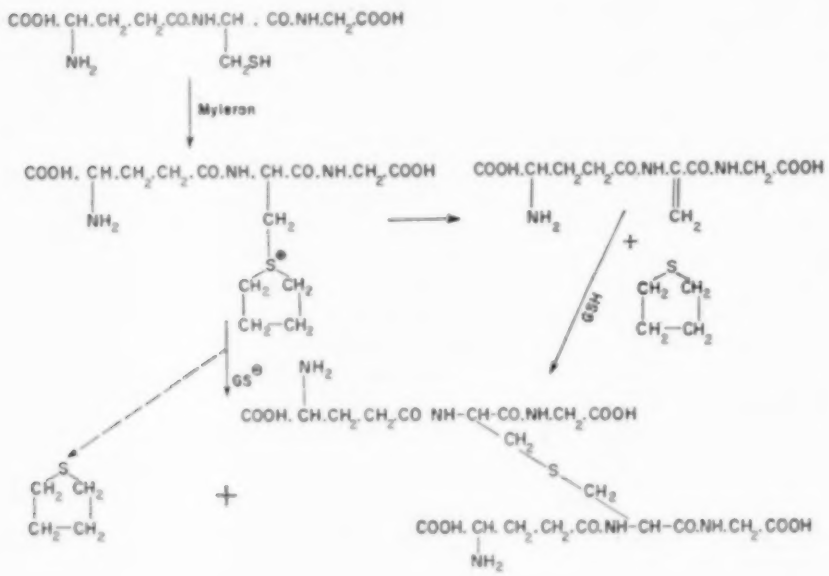


(where R and R′ represent H and OH, respectively, or amino acid, peptide, or protein residues, and $X^{\ominus}$ represents a nucleophilic reagent such as an ionized thiol).

In the first case the remaining residue would be a dehydropeptide (or dehydroprotein) (B), and in the latter a new saturated amino acid or amino acid chain would appear in place of the original cysteinyl moiety (C).

The chemical reactivity and stability of model dehydropeptides has been studied by Greenstein[15] and Nicolet[16] and it seems likely that in the body they may undergo either destruction by dehydropeptidases[17] or addition reactions with compounds such as thiols and amines. In each case new amino acids would appear in the molecule in place of the original cysteinyl moiety.

One such change which has been shown to occur very readily *in vitro* is the replacement of the cysteinyl moiety of glutathione by bound lanthionine (IX) after Myleran treatment (cf. Part II [6]). This transformation could have resulted by either of the two mechanisms outlined ((i) and (ii)), and indicated in the following scheme.



The reaction which might be general for peptides and proteins may be regarded as either the incorporation of a potential antimetabolite or as the cross-linking of two peptide chains.

Changes such as those indicated could be regarded as a type of chemical mutation, and attempts are now in progress to determine the nature of the modifications which occur *in vivo* following Myleran administration.

Other bis-aklylating agents such as the sulphur and nitrogen mustards or epoxides could conceivably undergo *in vivo* sulphonium ion formation with cysteine moieties in an analogous manner, and in this connexion studies with dichloroethylaniline have shown that a reaction of this type does occur *in vitro*.

Acknowledgements—We wish to thank Professor A. Haddow and Dr. L. A. Elson for their interest in the work. We are also grateful to Miss M. Moreton and Miss R. Cotter for technical assistance.

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DIE HEMMUNG DER KRISTALLISIERTEN D-GLYCERALDEHYD-3-PHOSPHAT DEHYDROGENASE AKTIVITÄT DURCH PHENOTHIAZINE UND AMINOKETONE

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Abstract—Various phenothiazine and aminoketone derivatives inhibit the activity of PGAD crystallized from swine muscle at a concentration of between 10^{-5}–10^{-6} M. The inhibition disappears when incubated with cystein and develops only during illumination.

Es IST aus der Literatur bekannt, dass Phenothiazine und Aminoketone *in vitro* die oxydative Phosphorylierung Gehirn-, und Lebermitochondrien hemmen.[1–4] Diese Hemmung ist mit Hilfe von Cystein auf zu heben.[5] Nach unseren früheren Versuchen beruht die *in vitro* beobachtete Wirkung teilweise wahrscheinlich auf einer Hemmung der SH Gruppen enthaltenden Fermente und Kofermente des phosphorylierenden Systems (Hexokinase, Brenztraubensäre-, Ketoglutarsäureoxydase).[6, 7]

Zur Untersuchung des genaueren Wirkungsmechanismus haben wir unsere Versuche mit kristallisierter D-Glyceraldehyd-3-Phosphat Dehydrogenase (PGAD) durchführt, welche essentielle SH Gruppen enthält.[8]

METHODE

Die Aktivität der aus Schweinemuskel isolierte PGAD wurde mit Hilfe der optischen Methode von Warburg bestimmt.[8] Der Versuch wurde folgenderweise durchführt: Das Fermenteiweiss (30 μg = 4,8 $\times$ 10^{-10} M) wurde 0–30 Min lang mit den untersuchten Substanzen auf Zimmertemperatur inkubiert, deren Konzentration 1,6 $\times$ 10^{-5} M war. Aus diesem wurde 0,1 ml dem Reaktionsgemisch zugefügt. In einigen Experimenten wurde das Ferment und die geprüften Präparate in Gegenwart von Cystein inkubiert (Endkonzentration 1 $\times$ 10^{-3} M). In solchen Fällen wurde auch der Kontrollprobe Cystein zugefügt, da Cystein die Aktivität der untersuchen Fermentpräparate um 20–30% hebt. Die Beleuchtung wurde bei Tageslicht in 5 ml Flüssigkeit enthaltenden Proberöhren durchgeführt.

Folgende Phenothiazin- und Aminoketonderivate wurden untersucht:

(1) Chloropromazin (Chlor-3-(dimethylamino-3'-propyl)-10-phenothiazin). (a) Largactil (Specia). (b) Hibernal (Egyesült Gyógyszer és Tápszergyár).

(2) Promethazin(N-(2'-dimethylamino-2'-methyl) (aethyl)-10-phenothiazin). (a) Phenergan (Specia). (b) Pipolphen (Egyesült Gyógyszer és Tápszergyár).

(3) Diaethazin(N-(2'-diaethylamino-aethyl)-phenothiazin). Latibon (Bayer).

(4) Promazin-(N-(3′-dimethylamino)-propyl-phenothiazin). Talofen (Pierrel).

(5) Prochlorpremazin-(Chlor-3(N-methyl-4′′-piperazinyl)-3′propyl)-10-phenothiazin). Stemetil (Specia).

(6) NA-86, 2-piperidino-methyl-tetralon-1-HCl.

(7) N-664, 2-piperidino-methyl-7-aethyl-tetralon-1-HCl.

(8) N-694, 2-morpholino-methyl-7-aethyl-tetralon-1-HCl.

(9) N-695, 2-piperidino-methyl-4,6,7-trimethyl-tetralon-1-HCl.

(10) N-697, 2-piperidino-methyl-7-chlor-tetralon-1-HCl.

(11) NA-65, 1-piperidino-3-phenylpropan-3-on-HCl.

(12) NA-127, 1-piperidino-3-phenylpropan-3-ol-HCl.

Die Aminoketonderivaten 6–12 wurden von Dr. K. Nádor (Abteilung für Arzneimitellforschung des Institutes für experimentelle Medizinsche Forschung der ungarischen Akademie der Wissenschaften) synthetisiert und zur Verfügung gestellt wofür wir auch an dieser Stelle unseren Dank ausdrücken.

ERGEBNISSE

TABELLE 1. CHLORPROMAZINHEMMUNG DER PGAD AKTIVITÄT IN VERSCHIEDENEN KONZENTRATIONEN, NACH VERSCHIEDENER INKUBATIONSZEIT UND BELEUCHTUNG

Untersuchte Substanz	Konzentration (M)	Inkubationszeit (Min)	Aktivität nach 30 Sek.	
			ΔE_{340}	in % der Kontrolle
Kontrollprobe	—	—	0,210	100
Largactil	$1{,}6 \times 10^{-5}$	2	0,145	70
Largactil	$1{,}6 \times 10^{-5}$	10	0,095	46
Largactil	$1{,}6 \times 10^{-5}$	20	0,020	10
Largactil	$1{,}6 \times 10^{-5}$	30	0,000	0
Largactil	$3{,}15 \times 10^{-6}$	25	0,140	70
Largactil	$1{,}6 \times 10^{-6}$	15	0,150	83
Largactil	$1{,}6 \times 10^{-6}$	60	0,085	40
Chlorpromazin	$1{,}6 \times 10^{-5}$	30 beleuchtet	0,080	38
Chlorpromazin	$1{,}6 \times 10^{-5}$	30 in Dunkeln	0,215	102
Chlorpromazin	$1{,}6 \times 10^{-5}$	30 beleuchtet, nachher im Dunkeln mit PGAD 30′ inkubiert	0,220	105

Aus Tabelle 1 ist es sichtbar, dass $1{,}6 \times 10^{-5}$ M Largactil die Fermentaktivität nach 30 Min vollkommen hemmt. Aus den Versuchen mit Chlorpromazin geht hervor, dass die Chlorpromazinhemmung im Dunkeln nicht zu Stande kommt, und dass die Beleuchtung gleichzeitig mit der Inkubation mit PGAD zur Auslösung der hemmenden Wirkung notwendig ist, auf welche Erscheinung wir noch später zurückkehren werden.

In den weiteren Versuchen haben wir die verschiedenen Phenothiazinderivative und Aminoketone unter gleichen Versuchsbedigungen und Konzentrationen ($1{,}6 \times 10^{-5}$) miteinander verglichen. Das Ferment wurde 20 Min mit den untersuchten Präparaten in Abwesenheit und in Gegenwart von Cystein (1×10^{-3} M) inkubiert. (Tabelle 2).

Unsere weitere Versuche zeigen, dass die hemmende Wirkung der Phenothiazine und Aminoketone auf die Aktivität der PGAD zwar mit Cystein aufzuheben ist,

aber nicht auf der direkten Blockierung der SH Gruppen beruht. Das Spektrum des Chlorpromazins zeigte im Gegensatz zu anderen Angaben[9] keine ausgesprochene Abweichung in Gegenwart von Cystein. Dagegen kommt eine Veränderung im Spektrum des Chlorpromazins vor bei Beleuchtung mit Abnahme des Adsorptionsmaximums bei 256 mμ unter der Bildung eines neuen Adsorptionsmaximums bei 275 mμ. Die Veränderung wird von PGAD katalysiert. DPN freie PGAD übt diese katalysierende Wirkung nicht aus (Tabelle 3).

TABELLE 2. PGAD AKTIVITÄTSHEMMUNG VERSCHIEDENER PHENOTHIAZIN UND AMINOKETONDERIVATE IN GEGENWART UND ABWESEN VON CYSTEIN

Untersuchtes Präparat	Aktivität nach 30 Sek.			
	Ohne Cystein		Mit Cystein	
	ΔE_{340}	in % der Kontrolle	E_{340}	in % der Kontrolle
Kontrollprobe	0,270	100	0,340	100
Chlorpromazin	0,145	54	0,335	98
Largactil	0,081	30	0,323	95
Hibernal	0,030	10	0,290	85
Phenergan	0,081	30	0,335	98
Pipolphen	0,085	35	0,330	96
Latibon	0,140	52	0,310	91
Talofen	0,115	42	0,345	102
Stemetil	0,105	39	0,335	98
Na-86	0,100	37	0,320	94
N-664	0,095	35	0,325	95
N-694	0,130	48	0,330	97
N-695	0,095	35	0,335	98
N-697	0,145	54	0,340	100
NA-65	0,150	55	0,295	87
NA-127	0,270	100	0,350	103

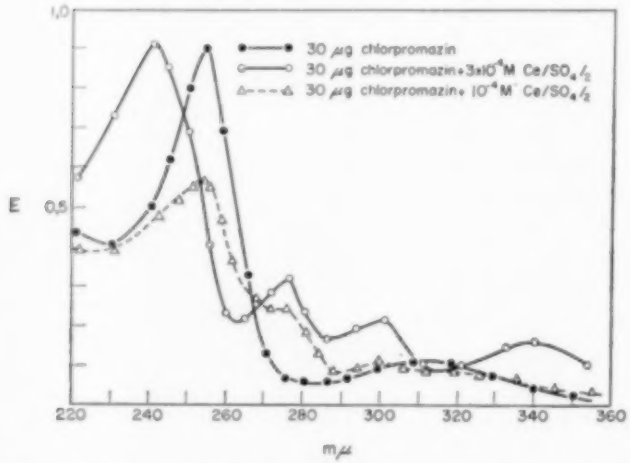

ABB. 1. Adsorptionsspektrum der Oxydation des Chlorpromazins mit Hilfe von $Ce(SO_4)_2$.

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Sämtliche spektrophotometrische Proben wurden gegen, Eiweiss bzw. Cystein gemessen. Das beleuchtete Produkt ist nicht mit dem Chlorpromazinsulfoxyd identisch[16] da es kein Maximum bei 240 mμ erweist. Bei der partiellen Oxydation des Chlorpromazins mit Hilfe von $Ce(SO_4)_2$ haben wir jedoch eine ähnliche intermediäre Verbindung wahrgenommen (Abb. 1).

TABELLE 3. VERÄNDERUNGEN IM ADSORPTIONSSPEKTRUM DES CHLORPROMAZINS WÄHREND BELEUCHTUNG, INKUBATION MIT PGAD UND CYSTEIN

Wellenlänge (mμ)	Chlorpromazin (10^{-5} M)			Chlorpromazin + PGAD (10^{-5} M) (10^{-9} M)		
	Zeit der Beleuchtung (Min)			Zeit der Beleuchtung (Min)		
	0	15	30	0	15	30
256	0,85	0,75	0,65	0,85	0,58	0,45
275	0,02	0,03	0,05	0,025	0,10	0,20
240	0,40	0,38	0,37	0,42	0,42	0,40
	Chlorpromazin + Cystein (10^{-5} M) (10^{-4} M)			Chlorpromazin + DPN freie PGAD (10^{-5} M) (10^{-9} M)		
256	0,84	0,78	0,75	0,87	0,76	0,65
275	0,02	0,02	0,03	0,02	0,03	0,05
240	—	—	—	0,40	0,39	0,40

DISKUSSION

Als mechanismus der PGAD Aktivitätshemmung der Phenothiazine kann also nicht nur die Bindung der SH Gruppen in Frage kommen, sondern auch eine photosensible Redoxveränderung der Phenothiazine, dass durch Cystein gehemmt wird. Eine ähnliche Veränderung hat auch Cavanaugh bemerkt[10] bei der Peroxydase Oxydation der Phenothiazine. Nach Literaturangaben[11] und nach unseren Erfahrungen[12] hemmen Phenothiazine auch andere Dehydrogenasen. Bei der Hemmung der PGAD Aktivität konnten wir jedoch nicht Veränderungen in der Zahl der SH Gruppen beweisen.[13] Diese Angaben und die Versuche japanischer Autoren[14] dass die Phenothiazine die Aminosäureoxydasenaktivität durch kompetitive Hemmung der Flavinnukleotide aufheben, haben uns auf die Annahme des obenerwähnten Mechanismus geführt. Es kann sich also bei der PGAD Hemmung der Phenothiazine um eine Redoxveränderung oder um eine Bildung eines wirksamen Intermediärs handeln. Gegen die letztere Annahme spricht jedoch, dass das beleuchtete Produkt während einer Inkubation im Dunkeln die PGAD Aktivität nicht hemmt. Es konnte aber während der Beleuchtung auch eine Polymerisation des Chlorpromazins stattfinden, welche die reaktiven Gruppen bindet.[15] Die Entscheidung dieser Frage ist noch Aufgabe weiterer Versuche.

"Die zwischen dem Chlorpromazin, Largactil und Hibernal gefundene Differenze in der Hemmung lassen sich dadurch erklären, dass die Präparate Largactil und Hibernal verschiedene, stabilisierende Substanzen enthalten (z.B. Natriumhydrocarbonat, Hydrochinon, Natriumsulfit, Kaliumpyrosulfat u.s.w.) Diese Reduktionsmittel bewaren die Phenothiazine einigermassen vor frühzeitiger Oxydation und Polymerisation. Dagegen nimmt das Maximum des Adsorptionsspektrum des Chlorpromazins schon nach einigen Minuten nach der Lösung ab, wie es aus

dem Kontrollversuch der Tabelle 3 auch sichtbar ist. Mit Chlorpromazinsulfoxyd beobachteten wir nur eine 10–20%-ige Hemmung in derselben Konzentration."

ZUSAMMENFASSUNG

Verschiedene Phenothiazin- und Aminoketonderivate hemmen die Aktivität der aus Schweinemuskel kristallisierter PGAD in einer Konzentration zwischen 10^{-5}–10^{-6} M. Die Hemmung wird in Gegenwart von Cystein aufgehoben, und entsteht nur nach Beleuchtung.

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SUPPRESSION OF UPTAKE OF L-ARGININE-U-^{14}C INTO CHROMATOGRAPHICALLY SEPARATED CATIONIC NUCLEAR PROTEINS OF TISSUES OF TUMOUR-BEARING RATS BY 5-BIS (2-CHLOROETHYL) AMINOURACIL*

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Abstract—At varying time periods ranging from 2 to 24 hr after pre-treatment with 4 mg of 5-bis (2-chloroethyl) aminouracil per kg, rats bearing the Walker 256 carcinosarcoma were injected intraperitoneally with 10 μc of L-arginine-U-^{14}C. One hour after injection of the tracer, the acid-soluble nuclear proteins of tumor, liver and spleen were isolated and chromatographed on carboxymethylcellulose columns. Comparison of the control values with the results obtained 4 hr following administration of the drug indicates that, in the spleen, the specific activities of the proteins of those radioactive protein peaks which have been termed RP1-A, RP2-A, RP3-A, RP4-A and RP5-A, were reduced to 14, 28, 22, 0 and 36 per cent, respectively, of the control values. In the tumor, 24 hr after administration of the drug, the specific activities of the proteins of radioactive protein peaks RP3-A and RP4-A were reduced to 24 and 38 per cent of the control values, respectively. The specific activities of the proteins of the liver were not significantly changed at any of the times they were examined. These data provide evidence that the effects of aminouracil mustard on the incorporation of this amino acid into nuclear proteins are, in part, specific. The data also provide an indication of the marked sensitivity of metabolism of nuclear proteins of the spleen to the effects of this drug.

RECENT studies from this laboratory[1] have indicated that the nitrogen mustard of uracil, 5-bis (2-chloroethyl) aminouracil markedly suppresses the incorporation of L-arginine-U-^{14}C into the nuclear proteins of the Walker tumor. Although the inhibition of uptake of the isotope was greatest in the acid-insoluble nuclear proteins, the inhibition of incorporation of the L-arginine-U-^{14}C into the cationic nuclear proteins (histones) was also very marked. The latter proteins have recently been found to contain a variety of chromatographically separable components,[2] some of which are different in tumors from those found in other tissues. In view of the availability of reproducible methods for chromatographic analysis of the cationic nuclear proteins, the question of specificity was examined in the attack of the mustard as an inhibitor of amino acid incorporation into the individual components of the cationic nuclear

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† Smith, Kline and French Foundation Summer Fellow.

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proteins. In the present studies, rats bearing the Walker 256 carcinosarcoma were treated with 5-bis(2-chloroethyl) aminouracil[1] and 1 hr before the termination of the experiment, L-arginine-U-^{14}C was injected intraperitoneally. The liver, spleen and tumor were treated as previously described for extraction and chromatographic analysis of the cationic nuclear proteins.[3] The data obtained indicate that although the drug exerts a suppressive effect on the incorporation of L-arginine-U-^{14}C into the cationic nuclear proteins, the effects on the incorporation of this amino acid into cationic nuclear proteins are in part specific.

MATERIALS AND METHODS

The animals used in these experiments were male rats weighing 180–220 g, obtained from the Holtzman Rat Company (Madison, Wisconsin). The Walker 256 carcinosarcoma was implanted from 7 to 10 days prior to the experiment.* In the present studies, 5-bis (2-chloroethyl) aminouracil,† dissolved in dimethylformamide, was administered intraperitoneally as a single dose of 4 mg/kg, to rats bearing the Walker 256 carcinosarcoma.[1] At a specified time interval following the administration of the drug, 10 μc of L-arginine-U-^{14}C (Nuclear-Chicago) with a specific activity of 5·3 mc/mmole was injected intraperitoneally. One hour after the administration of the labeled arginine the rat was anesthetized by intraperitoneal injection of sodium thiopental (50 mg/kg), and exsanguinated by aspiration of blood from the aorta. The tissues were rapidly excised, placed in ice-cold 0·25 M sucrose, and homogenized in isotonic sucrose (1:9 w/v) in the cold room (4 °C). The nuclei of tumor and liver were isolated by differential centrifugation.[3] For the nuclear preparation of the spleen the precipitate collected at 600 *g* was utilized. The cationic nuclear proteins were extracted from the nuclei with 0·25 N HCl for 30 min. The acid extract was separated from the insoluble residue by centrifugation at 600 *g* and clarified by centrifugation at 100,000 *g* for 30 min. The supernatant solution was dialyzed overnight against 0·5 N acetic acid at 4 °C and chromatographed in the cold room (4 °C) on carboxymethylcellulose columns with 1 N and 8 N formic acid as the eluting agents.[2] The protein concentration in the effluent fractions was measured by determination of the optical density of the solutions at 280 mμ in a Beckman DU spectrophotometer. Aliquots of each effluent fraction were pipetted onto stainless steel planchets, dried by warm air on a rotating turntable and assayed for radioactivity in a Nuclear-Chicago automatic counting system.

Two types of control experiments were carried out. In one group the animals were injected with dimethylformamide (1 ml/kg). Animals in the other group were untreated. Since the results were not significantly different between these two groups, the data have been pooled and designed as the "control".

RESULTS

Specific activity of protein of unfractionated acid extract of nuclei

Fig. 1 presents the specific activities (counts/min per E_{280}) of the protein of the whole acid-extract of the nuclear preparations of the Walker tumor and spleen. In the spleen the incorporation of labeled arginine into the acid-soluble nuclear proteins

* We are indebted to Mrs. Dolores C. Firszt for transplantation of the Walker 256 carcinosarcoma.
† 5-Bis (2-chloroethyl) aminouracil was generously supplied by the Upjohn Company, Kalamazoo, Michigan, through the courtesy of Dr. Harold G. Petering.

was inhibited by 72 per cent with 4 hr after administration of the drug. Progressively greater inhibition was noted at succeeding time intervals. In the tumor no inhibition of labeling was found up to 12 hr after administration of the drug; however, by 18 and 24 hr the incorporation of label was reduced by 26 and 49 per cent, respectively, of control values. The incorporation of radioactive arginine into the proteins of the

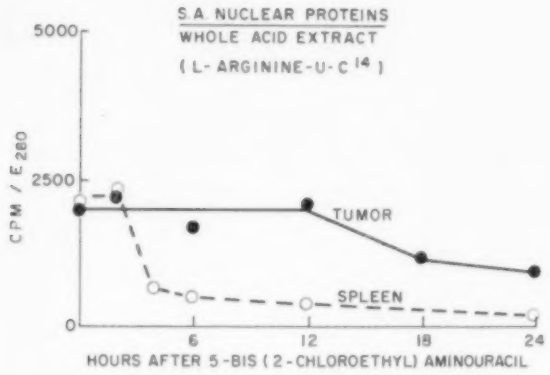

FIG. 1. Kinetics of the effects of the aminouracil mustard on incorporation of L-arginine-U-^{14}C into the whole acid-soluble nuclear protein extracts of Walker tumor and spleen. Each point represents the average of from two to four experiments. The values expressed as counts/min per $E_{280} \pm$ the standard error for the control and after 2 hr, 6 hr, 12 hr, 18 hr and 24 hr, were 1980 ± 30, 2270 ± 670, 1660 ± 320, 2120 ± 430, 1450 ± 310 and 1000 ± 190, respectively, for the tumor. The values for the control and after 2 hr, 4 hr, 6 hr and 12 hr, were 1700 ± 280, 2320 ± 480, 650 ± 130, 560 ± 160 and 440 ± 80, respectively, for the spleen.

whole acid extract of the liver did not differ significantly from the control values at 24 hr following administration of the drug.

Chromatographic patterns

The patterns for protein distribution in the chromatograms of the cationic nuclear proteins were the same in these studies as those reported previously.[2] The pattern for elution of the radioactive protein was different when arginine-U-^{14}C was used as the radioactive precursor, as compared to the pattern when lysine-U-^{14}C was employed.[2] With samples from spleen, five radioactive peaks were eluted from the column; these have been designated* as RP1-A, RP2-A, RP3-A, RP4-A and RP5-A (Fig. 2). Of these, RP1-A, RP4-A and RP5-A were eluted simultaneously with protein peaks *A*, *E* and *F*, respectively, while RP2-A was eluted with the first peak of the split peak labeled protein peak *B*. In samples of the Walker tumor, only RP3-A and RP5-A were present (Fig. 3). In samples of liver, RP2-A, RP3-A, and RP5-A were found; RP2-A

* The abbreviation used for the various radioactive peaks is RP1-A, etc. (first radioactive peak obtained with L-arginine-U-^{14}C as the tracer). Inasmuch as the positions of the peak tubes varied ± 3 fractions from the mean positions indicated in the chromatograms,[2] it is apparent that many criteria are required for adequate comparisons of the proteins of different tissues other than volume of acid required for elution. Studies are now in progress on the structures of the proteins of the various chromatographic peaks which will provide evidence as to their identity and differences.

and RP3-A were eluted just before and just after the peak of protein peak *B* (Fig. 4). RP5-A was eluted with protein peak *E*.

Kinetics of the effects of the aminouracil mustard on incorporation of L-*arginine-U-*^{14}C *into various chromatographic fractions*

The kinetics of the inhibitory effects of the aminouracil mustard on the incorporation of labeled arginine into various chromatographic fractions of the Walker tumor and

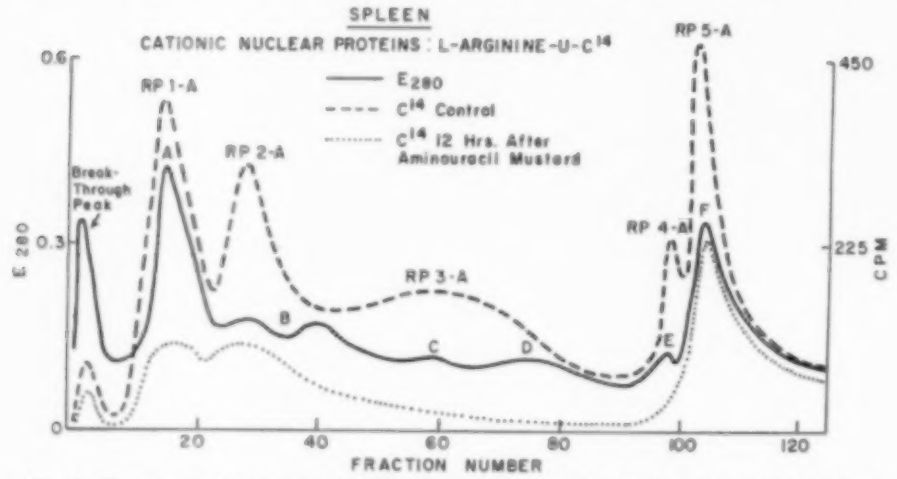

FIG. 2. Chromatographic patterns of protein concentration and radioactivity for the acid-soluble nuclear proteins of spleen. The radioactivity patterns represent both the untreated animal and the animal 12 hr after administration of the drug. Radioactivity and protein concentration were assayed in each effluent fraction and hence points are omitted from the chromatograms. The data are averages of from two to four experiments.

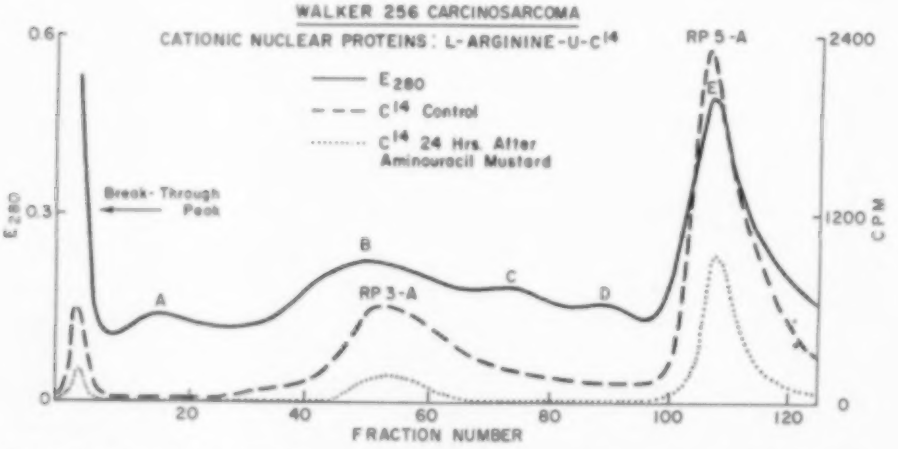

FIG. 3. Chromatographic patterns of protein concentration and radioactivity for the acid-soluble nuclear proteins of the Walker 256 carcinosarcoma. The radioactivity patterns represent both the untreated animal and the animal 24 hr after administration of the drug. Also see legend for Fig. 2.

spleen are presented in Figs. 5 and 6. In the *Walker tumor* the inhibition of incorporation of label* into fractions RP3-A and RP5-A follow a parallel course. Within 2 hr after administration of the drug, the specific activities of RP3-A and RP5-A were 67 and 73 per cent of the control values, respectively. By 24 hr after administration of the drug the specific activities were 24 and 38 per cent of control values of RP3-A

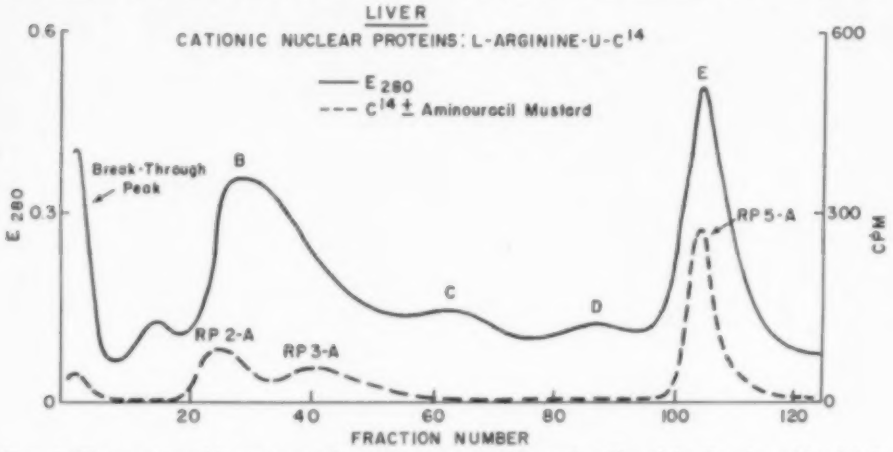

FIG. 4. Chromatographic patterns of protein concentration and radioactivity for the acid-solubel nuclear proteins of liver. Also see legend for Fig. 2.

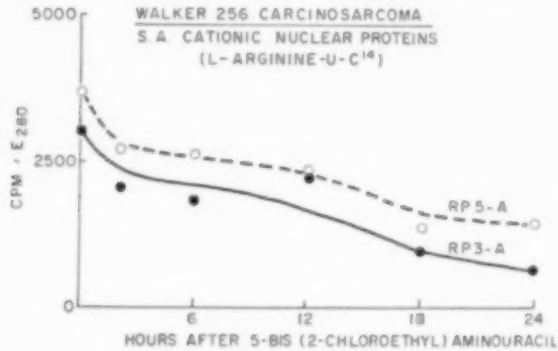

FIG. 5. Kinetics of the effects of the aminouracil mustard on incorporation of L-arginine-U-^{14}C into chromatographically isolated peaks of the acid-soluble nuclear proteins of the Walker tumor. Each point represents the average of from two to four experiments. The values expressed as counts/min per E_{280} ± the standard error for the control and after 2 hr, 6 hr, 12 hr, 18 hr and 24 hr, were 3020 ± 500, 2030 ± 790, 1820 ± 340, 2200 ± 60, 970 ± 520 and 610 ± 70, respectively, for peak RP3-A, and 3710 ± 450, 2700 ± 720, 2630 ± 670, 2320 ± 290, 1360 ± 540 and 1450 ± 340, respectively, for peak RP5-A.

* The specific activity of each radioactive protein fraction studied is expressed as counts/min per E_{280}. In order that these values be readily convertible to conventional specific activity values of counts/min per mg of protein, the eluted fractions comprising each defined protein peak eluted from a single column were pooled, and the E_{280} values and total mg of protein determined. Each value represents the average of two or three experiments and is presented as mg dry protein/E_{280} ± the standard error. The values found for the tumor were 1·53 ± 0·22 and 1·31 ± 0·12, for peaks *B* and *E*, respectively; for the spleen, 0·18 ± 0·03, 1·35 ± 0·15, and 1·11 ± 0·22, for peaks *A*, *B*, and *F*, respectively; for the liver, 2·00 ± 0·06, and 2·13 ± 0·03 for peaks *B* and *E*, respectively.

and RP5-A, respectively (Table 1). In the *spleen* a marked inhibition of labeling of the various peaks was found by 4 hr following the administration of the drug (Fig. 6). The evidence for intracellular selectivity of action of this mustard is found in Table 1. The specific activities of proteins in RP1-A, RP2-A, RP3-A and RP5-A were 14, 28, 22 and 36 per cent of control values, respectively. The fraction termed RP4-A was

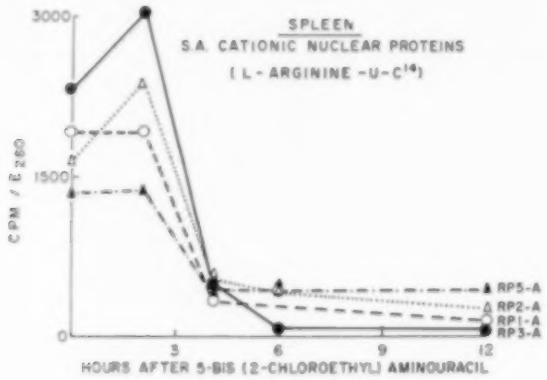

Fig. 6. Kinetics of the effects of the aminouracil mustard on incorporation of L-arginine-U-^{14}C into chromatographically isolated peaks of the acid-soluble nuclear proteins of spleen. Each point represents the average of two to three experiments. The values expressed as counts/min per E_{280} ± the standard error for the control and after 2 hr, 4 hr, 6 hr and 12 hr, were 1920 ± 130, 1920 ± 160, 330 ± 70, 410 ± 30, and 130 ± 1, respectively, for peak RP1-A, 1630 ± 600, 2380 ± 60, 540 ± 190, 420 ± 40 and 240 ± 50, respectively, for peak RP2-A, and 1350 ± 230, 1380 ± 200, 480 ± 100, 500 ± 70 and 430 ± 70, respectively, for peak RP5-A. Values for peak RP3-A for the control and after 2 hr and 4 hr were 2300 ± 580, 3050 ± 200 and 470 ± 90, respectively. The control value for peak RP4-A was 1890 ± 500.

Table 1. Relative incorporation of L-arginine-U-^{14}C into chromatographic peaks of acid-soluble nuclear proteins of tumor and spleen after administration of 4 mg of the aminouracil mustard per kg, as compared to control values

(The data are expressed in terms of percentage of control values ± the standard error. The number of experiments is shown in parentheses. The standard error, E, is $\sqrt{[\Sigma d^2/n(n-1)]}$.

Fraction	Spleen 4 hr after administration of the drug	Tumor 24 hr after administration of the drug
	% control	% control
RP1-A	14·2 ± 5·0 (3)	
RP2-A	27·5 ± 12·4 (3)	
RP3-A	21·9 ± 2·7 (3)	24·0 ± 2·9 (3)
RP4-A	0 (3)	
RP5-A	36·3 ± 1·7 (3)	38·4 ± 1·9 (3)

not found in samples of spleen at periods of 2 hr or more following the administration of the drug (Table 1).

Table 2 presents the comparative specific activities of the radioactive protein peaks obtained in chromatograms of the acid-soluble nuclear proteins of livers of control animals and livers from animals 24 hr after administration of the 5-bis (2-chlorethyl) aminouracil. The specific activities of the proteins were somewhat increased in the whole acid-extract and in RP5-A in comparison with control values.*

TABLE 2. THE INCORPORATION OF L-ARGININE-U-^{14}C INTO ACID-SOLUBLE NUCLEAR PROTEINS OF LIVER AFTER ADMINISTRATION OF 4 MG OF THE AMINOURACIL MUSTARD PER KG

(The data are presented as counts/min per E_{280} ± the standard error. The number of experiments is shown in parentheses. The standard error, E, is $\sqrt{[\Sigma d^2/n(n-1)]}$.

Fraction	Control		24 hr after administration of the drug	
Whole acid extract	475 ± 90	(3)	681 ± 108	(2)
RP2-A	676 ± 168	(3)	630 ± 203	(2)
RP3-A	642 ± 89	(3)	656 ± 188	(2)
RP5-A	840 ± 157	(3)	1088 ± 174	(2)

DISCUSSION

The data obtained in this study indicate partial intracellular specificity of action of the agent, 5-bis (2-chloroethyl) aminouracil. Such a result represents an advance in chemotherapy, since intra-tissue specificity of effect has been sought[4, 5] both from empirical and from theoretical considerations.[4–7] While the aminouracil mustard has been very effective against certain neoplasms of animals, it has not been therapeutically superior to other alkylating agents in human cancer chemotherapy.[8–11] However, therapeutic trials have not yet been completed. Nonetheless, any indication of intracellular specificity of action is particularly important from the standpoint of the endeavors of biochemists to establish the key reactions which characterize the neoplastic transformation in terms of alterations in the structure or activities of nucleoproteins.[2, 12] The nitrogen mustard of aminouracil has been used in these studies because the data on kinetics of inhibition of nuclear protein biosynthesis indicated a separation of biochemical events with respect to the time sequence of inhibition of labeling of various intracellular proteins.[1] Studies now in progress with a variety of antitumor agents have shown that this time sequence of inhibition is best demonstrated with the aminouracil mustard, which is therefore a useful biochemical tool.

The availability of methodology for the separation of components of the nuclear proteins has provided some evidence for differential effects of the aminouracil mustard on the anabolism of specific cationic nuclear proteins. However, these data are at present preliminary in the sense that as yet the task remains of isolation, purification and characterization of the proteins in the fractions obtained. Such studies are now in

progress with particular emphasis on those proteins in the area of that fraction of the proteins of the Walker tumor which has been termed RP2-L. If the proteins of this peak prove to be unique to the tumor, it might be possible to devise drugs which exert as marked an effect on this fraction as was found with aminouracil mustard and the fraction termed RP4-A of the spleen.

The data obtained in this study emphasize the remarkable effects of aminouracil mustard on the metabolism of the nuclear proteins of the spleen. Both the rate and extent of the inhibition of amino acid incorporation into the cationic nuclear proteins of the spleen were greater than that found for the tumor. One possible explanation for the marked effect of the aminouracil mustard on the proteins of the spleen is a selective affinity for the lymphocytes or their precursors in the spleen. Although greater suppression was observed for the chromatographic peaks eluted earliest in both tumor and spleen, it is apparent that specificity of site of action has not yet been achieved by the aminouracil mustard. This result, together with other data now being obtained for other antitumor agents, supports the possibility that the rate of amino acid incorporation into nuclear proteins of the spleen might be used in examinations of new carcinotoxic agents for general cytotoxicity.

* Much of this research was carried out at the University of Illinois College of Medicine to which we are indebted for the facilities and the opportunity to carry forward these studies.

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THE REDUCTION OF COENZYME Q_{10} BY AN ANTICOAGULANT SENSITIVE ENZYME FROM DOG LIVER*

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Abstract—An enzyme obtained from dog liver, was found to catalyse the reduction of coenzyme Q_{10} by DPNH and TPNH. This activity was inhibited by various anticoagulants, such as, Dicumarol, Cumachlor, Tromexan, Warfarin, Phenindione, and Sintrom.

INTRODUCTION

AN ENZYME which catalyzes the reduction of menadione (2-methyl-1:4-naphthoquinone), and vitamin K_1 by the reduced pyridine nucleotides, is inhibited by various anticoagulants.[1, 2] The partially purified enzyme obtained from dog liver also catalyzes the reduction of benzoquinone; thus, it was of interest to test coenzyme Q_{10}, a naturally occuring benzoquinone derivative, as a possible electron acceptor. The purpose of the present report is to describe some of the properties of this enzyme, with coenzyme Q_{10} as an electron acceptor, and the effect of anticoagulants on this system.

METHODS

Since coenzyme Q_{10} is not readily soluble in water, it is solubilized as follows: 4 mg of coenzyme Q_{10}† was suspended in 8·0 ml of 3·6 per cent BRIJ-35 (polyoxyethylene lauryl alcohol) and heated in a tightly stoppered 25-ml volumetric flask for about 16 hr at from 105 to 115 °C, providing a clear yellow solution which was filtered if necessary. Estimation of the amount of coenzyme Q_{10} solubilized made use of the extinction coefficient reported by Wolf *et al.*[3] In the oxidized and reduced form these preparations exhibited essentially the same spectrum as coenzyme Q_{10} in ethanol.[4] When chromatographed, the solubilized coenzyme migrated in the same manner as reference samples dissolved in ethanol.[5] These findings indicate that the coenzyme was not destroyed by the solubilization procedure and such preparations appeared to be suitable for the present studies. Samples containing BRIJ-35 without coenzyme Q_{10}, for use as controls and in check cells, were treated in the same manner.

The preparation of the purified enzyme and the general procedures used are those previously described.[2] The activity of the heat-labile enzyme was determined by observing the rate of oxidation of reduced diphosphopyridine nucleotide (DPNH) at 340 mμ. The standard reaction mixture used in the present studies contained 50 μmoles of

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† Kindly supplied by Dr. Karl Folkers.

imidazole buffer of pH 7·4, 0·1 μmole of DPNH, 0·002 μmole of flavin adenine dinucleotide (FAD), 0·1 μmole of coenzyme Q_{10}, water and enzyme to a final volume of 1·0 ml. The enzyme preparations used in the present studies, which had been purified from 100- to 300-fold, had the absorbance of a typical protein in the ultraviolet region of the spectrum but no measurable absorbance in the visible region. The activity of the purified enzyme without added flavin was from 10 to 20 per cent of that observed with saturating amounts of FAD or flavin mononucleotide. During purification of the enzyme, the ratio of activity with vitamin K_1 to coenzyme Q_{10} did not change appreciably with the different enzyme fractions tested.

RESULTS AND DISCUSSION

The activity of the purified enzyme was inhibited by various anticoagulants when coenzyme Q_{10} served as an electron acceptor; of the compounds tested, dicumarol was the most potent, inhibiting about 59 per cent at 10^{-5} M. Other anticoagulants tested, at a concentration of 10^{-4} M, caused the following percentage inhibitions: Cumachlor, 50; Tromexan, 45; Warfarin, 45; Phenindione, 25; Sintrom, 15. At higher concentrations of anticoagulant the inhibition was greater. Cations such as Mg^{2+}, Mn^{2+}, Fe^{3+}, Co^{2+}, Zn^{2+} and Ni^{2+}, at final concentrations of 10^{-4} M, did not appear to affect the rate of the enzymatic reaction when added to the standard assay system, while Cu^{2+}, at the same concentration, increased the rate several-fold.

In order to follow the reduction of coenzyme Q_{10} during the oxidation of DPNH, the reaction was also followed at 275 mμ, at which wavelength the reduced form of coenzyme Q_{10} has a lower absorbancy than the oxidized form.[4] Under the standard experimental conditions the reduced form was oxidized in the presence of air, therefore, the reduction was followed under anaerobic conditions using a modified system. As shown in Fig. 1, the oxidation of DPNH was accompanied by a decrease in absorption at 275 mμ, indicating reduction of coenzyme Q_{10}. On the basis of the change in absorbancy at 340 mμ, when the reaction reached a steady level, 0·12 μmole of DPNH, of the 0·55 μmole present, was oxidized by 0·14 μmole of coenzyme Q_{10}. This corresponds to about 1:1 relationship between the amount of coenzyme Q_{10} present and the amount of DPNH oxidized. The fact that benzoquinone also served as electron acceptor,[2] and that the absorbancy at 275 mμ decreased in an essentially stoichiometric manner, suggests that coenzyme Q_{10} might be reduced to the corresponding hydroquinone. Upon introduction of air to the system, the reduced coenzyme Q_{10} was reoxidized, as indicated by an increase in absorption at 275 mμ. The remaining DPNH was also oxidized, presumably as a result of a regeneration of oxidized coenzyme Q_{10}, which again acted as an electron acceptor. The rate of reoxidation of reduced coenzyme Q_{10} varied with different enzyme preparations; but whether this oxidation was catalyzed by trace metals or by coenzyme Q_{10} oxidase[6] in the enzyme preparation has not been determined. Under standard conditions, the rate of DPNH oxidation was greater than that illustrated in Fig. 1, because a modification of the standard assay was required in order that the spectral changes, occuring at 275 mμ, could be followed. DPN was identified as a product of the reaction by enzymatic regeneration with alcohol dehydrogenase. Chemically or enzymically reduced triphosphopyridine nucleotide also served as an electron donor in this system and, in this case, the product was identified by enzymatic procedures as oxidized triphosphopyridine nucleotide.

Although a number of quinones can serve as electron acceptors in this system, the identity of the natural electron acceptor is not certain and studies are in progress in order to clarify this point. However, it is of interest that this reduction of coenzyme Q_{10} was inhibited by various anticoagulants. In other studies the same anticoagulants were found to depress the respiration of rat liver slices,[7] and the relative order of

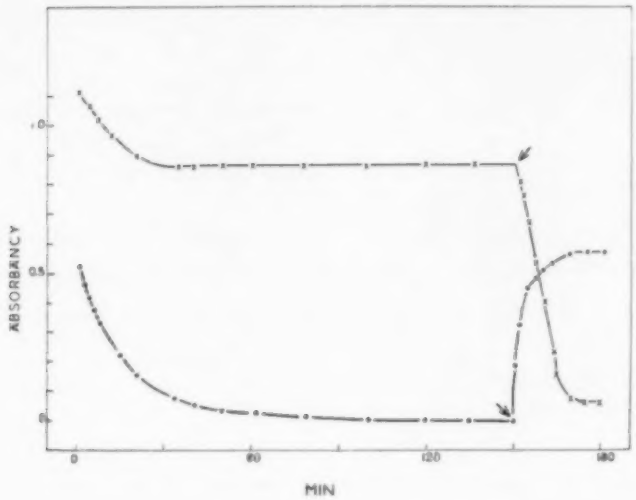

FIG. 1. Oxidation of DPNH and reduction of coenzyme Q_{10}. The reaction mixture was set up in anaerobic silica cells of the Thunberg type with 150 μmoles of imidazole buffer of pH 7·4, 0·005 μmole of FAD, 0·55 μmole of DPNH, and 0·14 μmole of coenzyme Q_{10} in a volume of 2·8 ml in the reaction chamber and 100 μg of enzyme in the side arm. After the cell was evacuated the reaction was started by tipping in the enzyme and the oxidation of DPNH was followed at 340 mμ while the reduction of coenzyme Q_{10} was followed at 275 mμ. In order to follow the reaction at 340 mμ and at 275 mμ, separate check cells were required for each wavelength. The check cell used at 340 mμ contained all components except DPNH and that used at 275 mμ contained all components except coenzyme Q_{10}. Air was introduced to the system at the time indicated by the arrows. ×——× 340 mμ; ●——● 275 mμ.

potency on the slices paralleled their inhibitory action on the purified enzyme. The present evidence does not permit any conclusion as to a causal relationship between the effects of these agents on the purified enzyme and their anticoagulant action.

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THE TOXIC ACTION OF SOME PHOSPHORUS ANTICHOLINESTERASES WITH CATIONIC GROUPS

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Abstract—The intravenous LD_{50} in rats and I_{50} to sheep AChE of several compounds of the type $(RO)_2PO{\cdot}S{\cdot}CH_2{\cdot}CH_2{\cdot}Z$ have been determined. Z represents SR, $\overset{\oplus}{S}RR'$ or, in one case, $\overset{\oplus}{N}R_3$, where the R's are alkyl groups. Special precautions had to be taken to get reliable values. The LD_{50} and I_{50} of the cationic compounds are very much lower than those of their uncharged analogues. Two of the sulphonium compounds are the most toxic phosphorus anti-AChE's reported. The results are discussed in terms of the mechanism of inhibition at the enzyme surface.

SEVERAL organophosphorus compounds have been described in which exceptionally high toxicity and anticholinesterase activity are associated with the presence of side-chain quaternary nitrogen groups. Some P:P-(fluoro)(methyl)phosphinylcholines[1] and phosphostigmines[2] are familiar examples. In this paper it is shown that some compounds with sulphonium groups in similar positions are even more toxic, estimated on a mole/kg basis, when given intravenously. They are compared with the closely related uncharged compounds from which they are derived, and with one quaternary nitrogen compound. The ten compounds studied are listed in Table 1, and can be represented by the general formula: $(RO)_2PO.S.CH_2.CH_2.Z$ where R is a methyl or ethyl group, and Z is a thioalkyl, dialkylsulphonium or trialkylammonium group.

The chemical properties of some of the compounds have been described.[3, 4] They are such that it is hard to determine accurate LD_{50}'s and I_{50}'s. The relevant features are therefore summarized here.

The thioalkyl compounds (nos. 1, 4 and 8 in Table 1) give sulphonium compounds slowly on storage and more rapidly in water.[3, 4] The sulphonium compounds are very much more toxic and active, and must be removed from the thioalkyl compounds immediately before the latter are used.

The structure of the sulphonium compounds follows from the method used to prepare them, and their behaviour during electrophoresis. In two cases, compound nos. 2 and 3, the structures have been confirmed by detailed analysis of the kinetics of reactions in which they are formed.[4] They can, however, only be got as dilute solutions. All attempts to separate them from solvent (usually water) resulted in their decomposition. Their concentrations had, therefore, to be estimated indirectly. Two methods were used. Relative concentrations of a given compound were calculated from the

capacities of different solutions to inhibit AChE. Thus if a solution must be diluted n-fold to produce 50 per cent inhibition of the enzyme under standard conditions, the concentration must be $n \times I_{50}$, whatever the I_{50} may be. Such relative concentrations are all that are needed for many kinetic studies. The only practicable way to determine absolute concentrations was by estimating the phosphorus contents of solutions. This

TABLE 1. THE COMPOUNDS ARE OF THE TYPE: $(RO)_2PO{\cdot}S{\cdot}(CH_2)_n{\cdot}Z$

No.	Compounds			LD_{50} (μg/kg)	I_{50} (M)	LD_{50}/I_{50}†
	R	n	Z			
1	Me	2	$S\overset{+}{E}t$*	54,000–72,000	$6{\cdot}5 \times 10^{-5}$	4·3
2	Me	2	$S\overset{+}{E}tMe$*	52–72	$3{\cdot}9 \times 10^{-9}$	6·5
3	Me	2	$S\overset{+}{E}t{\cdot}CH_2{\cdot}CH_2{\cdot}SEt$	4–5·2	$2{\cdot}1 \times 10^{-9}$	6·8
4	Et	2	SEt	1,700–2,380	$3{\cdot}5 \times 10^{-6}$	2·2
5	Et	2	$S\overset{+}{E}tMe$*	14–18	$4{\cdot}7 \times 10^{-9}$	13
6	Et	2	$S\overset{+}{E}t_2$*	10‡	$2{\cdot}6 \times 10^{-9}$	13
7	Et	2	$S\overset{+}{E}t{\cdot}CH_2{\cdot}CH_2{\cdot}SEt$	4·7–5·8	5×10^{-10}	28
8	Et	3	SEt	12,700–28,100	$1{\cdot}8 \times 10^{-5}$	3·9
9	Et	3	$S\overset{+}{E}tMe$	290	$1{\cdot}7 \times 10^{-7}$	5·9
10	Et	2	$N\overset{+}{E}t_2$	15–21	$4{\cdot}8 \times 10^{-9}$	13

* Values from Heath and Vandekar[3].
† To calculate this ratio LD_{50}'s were expressed in moles/kg.
‡ Determined on eight rats only.

method is only valid when the only phosphorus compound present is the anticholinesterase under investigation. For a specimen of compound no. 2 this assumption has been proved correct.[3] Compound no. 2 was subjected to electrophoresis, and the profile of the peak found both from the anti-AChE activities of the different fractions (the first method) and from their phosphorus contents (the second method). The two profiles were in excellent agreement. Compound no. 2 has a half-life in water of 14·3 days at 37 °C, so one can assume that any other sulphonium compound of similar stability will be equally pure. Compound no. 3, however, is much less stable (half-life 100 min at 37 °C), and must decompose to some extent during separation and storage. The methods used to estimate the purity of this and the other unstable compound (no. 7) are described in the next section.

COMPOUNDS AND METHODS

I_{50}'s *and* LD_{50}'s

The preparation and purification of specimens suitable for the determination of I_{50}'s and LD_{50}'s are described under "Compounds".

I_{50}'s were determined by Aldridge's method.[5] Known dilutions of the anti-AChE's were incubated with washed sheep red cells for 30 min at 37 °C, and the AChE remaining determined in a Warburg apparatus using acetylcholine (ACh) as substrate. The percentage inhibition increased with the concentration of inhibitor. For the dimethyl phosphate esters (1–3) the logarithm of the fraction of AChE remaining was not related linearly to the concentration. This is always the case with such esters, as the dimethylphosphorylated enzyme produced is reactivated fairly rapidly.[3, 6] The I_{50} concentration, i.e. the concentration inhibiting half the enzyme, was calculated as described by Heath and Vandekar.[3] The remaining esters gave a straight line graph as expected. I_{50}'s were reproducible within 5 per cent.

LD_{50}'s were determined on white rats (Porton Wistar Strain) of 150–200 g wt., given the compounds by the tail vein. Compound no. 1 was given neat, and compound nos. 4 and 8 in ethanol. The dose of ethanol was always less than 300 mg/kg. The rest were given in water. Survivors were observed for 24 hr, i.e. about 18 hr after the last death. LD_{50}'s and fiducial limits were calculated from the Tables given by Weil.[7]

Compounds

The thioalkyl compounds and the quaternary nitrogen compounds were received as gifts. The purity of the specimens of compound nos. 1 and 4 had been established.[3] Compound no. 8 was assumed to be equally pure. The quaternary nitrogen compound was crystalline, and its purity was not investigated.

Preparation of sulphonium compounds

The sulphonium compounds were prepared in low yields (1–10 per cent) by the reaction of the thioalkyl compounds with methyl or ethyl iodide or 2-bromoethylthioethane in ethanol at 37 °C for several hours:

$$(RO)_2PO{\cdot}S{\cdot}(CH_2)_n{\cdot}SEt + R'X \rightarrow (RO)_2PO{\cdot}S{\cdot}(CH_2)_n{\cdot}\overset{+}{S}EtR' + \overset{\ominus}{X}$$

where R'X is the alkyl halide. This is a standard method for the preparation of sulphonium compounds. The products were separated by paper electrophoresis.[3] Each compound moved in the electric field as expected of a cation. The compounds were washed from the paper, and stored at −30 °C. Their concentrations were estimated from the phosphorus content of the solutions determined as described by Heath and Vandekar.[3]

Purity of sulphonium compounds

The sulphonium compounds were contaminated with sodium acetate buffer, pH 5·3, and, of course, water. Buffer contaminants should not affect I_{50}'s or LD_{50}'s in the concentrations present. The purity of the compounds therefore refers to the relationship between the concentrations actually present, and those calculated from the phosphorus contents of the solutions. As the compounds were separated by electrophoresis, the only possible phosphorus impurities were other sulphonium compounds, and products formed during electrophoresis on that part of the strip extracted and during storage. The kinetics of decomposition can be used to show that only one sulphonium compound is present. Thus compound no. 2 decomposed in 10^{-4} N HCl according to first-order kinetics as far as the reaction was followed (about 90

per cent decomposition),[3] and the same was found for compound no. 7 in this investigation. The presence of sulphonium compounds of different stability would cause deviations from first-order kinetics. A similar test showed that compound no. 3 contained a little compound no. 2.[4] This is in accordance with the chemical properties of compound no. 1, from which compound no. 3 was prepared. Compound no. 1 forms compound no. 2 particularly readily by the reaction:

$$2(MeO)_2PO{\cdot}S{\cdot}CH_2{\cdot}CH_2{\cdot}SEt \rightarrow (MeO)_2PO{\cdot}S{\cdot}CH_2{\cdot}CH_2\overset{+}{S}EtMe + MeO{\cdot}P\overset{\ominus}{O}_2{\cdot}S{\cdot}CH_2{\cdot}CH_2{\cdot}SEt$$

Diethyl phosphate analogues undergo this reaction much more slowly.[3] It is therefore reasonable to assume that only solutions of compound no. 3 contained significant amounts of other sulphonium compounds.

Inactive phosphorus compounds were not present in compound no. 2 immediately after separation (see Introduction). Compound nos. 5, 6 and 9 were about as stable, and were therefore assumed to be pure also. Compound nos. 3 and 7 ($Z = \overset{+}{S}Et.CH_2.CH_2.SEt$) have half-lives in water of 100 and 85 min at 37 °C, respectively. (There are some incomplete experimental data and some theoretical reasons for believing that these rapid reactions are not hydrolyses but dissociation reactions:

$$-\overset{+}{S}Et{\cdot}CH_2{\cdot}CH_2{\cdot}SEt + \overset{\ominus}{X} \rightarrow -SEt + \overset{\ominus}{X}{\cdot}CH_2{\cdot}CH_2{\cdot}SEt)$$

Electrophoretic separation took several hours, so they could not have been pure. For compound no. 3 a previous investigation gives an estimate of purity.[4] From the phosphorus content and anti-AChE activity of a fresh preparation the I_{50} was calculated to be $6{\cdot}9 \times 10^{-9}$ M, assuming 100 per cent purity. Now in water compound no. 1 decomposes in two ways: according to first-order kinetics to give inert compounds, and according to second-order kinetics to give the sulphonium compound nos. 2 and 3.

The rate equation is thus:

$$dA/dt = -k_1A - k_2A^2$$

denoting the concentration of compound no. 1 by A. The k_2A^2 term can be determined from the rates at different concentrations, and hence the sum of the concentrations of compound nos. 2 and 3 calculated. The anti-AChE activities in the reaction mixtures due to compounds nos. 2 and 3 can be obtained separately, by taking advantage of the difference between their stabilities. The concentration of compound no. 2 can thus be calculated from its I_{50} and the activity present, and hence the concentration of compound no. 3 can be found by difference. This, with the anti-AChE activity of the solution gives the I_{50}. It was found to be $1{\cdot}4 \times 10^{-9}$ M. The value is not precise, as reactions involving compound no. 3 only contribute 5 per cent to the total rate constant. But this and another check show that the I_{50} is very unlikely to exceed about 2×10^{-9} M. The direct value was $6{\cdot}9 \times 10^{-9}$ M, so the purity was 30 per cent or less, 30 per cent has been assumed in this paper. No corresponding detailed analysis has been carried out for compound no. 7. During electrophoresis, however, about 75 per cent was decomposed, i.e. only 25 per cent of the anti-AChE put on the paper was recovered from it. As the paper was cut before extracting the specimen half-way

between the peak and the starting line (the rest of the paper was inactive), only inert phosphorus compounds formed in the second half of the separation should be recorded. This gives a purity of about 50 per cent, with a maximum of about 60 per cent, which has been assumed correct.

Decomposition during storage did not affect the results—for example, the anti-AChE activity of a solution of the least stable compound, compound no. 7, was determined twice, with 6 days storage at −30°C between determinations. The I_{50}'s agreed within 2·5 per cent.

Purification of thioalkyl compounds

Purification of the thioalkyl compounds immediately before use was necessary, as they contained sulphonium compounds. The formation of sulphonium compounds during purification had also to be avoided. The compounds were dissolved in chloroform or benzene, and the sulphonium compounds extracted with water. The non-aqueous solutions were then concentrated *in vacuo*. This also dried the compounds, as these solvents form azeotopes with water. To prepare specimens for LD_{50} determinations evaporation was carried out at less than 1 mm pressure at room temperature until the volume remained apparently constant. The products contained about 5 per cent of solvent, which could not be removed except by raising the temperature or greatly prolonging the evaporation, either of which led to the formation of sulphonium compounds. The compounds in the specimens were therefore estimated from determinations of phosphorus, and used within an hour, or stored at −30°C. The proportion of solvent was too small to affect LD_{50}'s. To prepare specimens for I_{50} determinations only small amounts were dissolved in the non-aqueous solvent, and the solvent was evaporated under about 15 mm pressure until the volume was reduced to 2–5 ml. Sufficient water was then added to give a solution of about 10^{-4} M, and the rest of the non-aqueous solvent evaporated. The compounds were thus never at high concentrations in aqueous solution, so avoiding the formation of sulphonium compounds, which are formed at rates proportional to the square of the concentrations of the thioalkyl compounds. The concentrations were estimated by phosphorus.

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RESULTS AND DISCUSSION

The I_{50}'s and LD_{50}'s are listed in Table 1. Those for compound nos. 3 and 7 are calculated assuming 30 and 60 per cent purity, respectively. The remaining compounds were assumed to be completely pure.

The higher the purity assumed the lower the biochemical activity and toxicity recorded, with the proviso discussed in the next paragraph. For all except compound nos. 3 and 7 there is every reason to suppose that the compounds were substantially pure, and that the main errors in the values are those in the determinations of I_{50}'s and LD_{50}'s: about ±5 per cent for the I_{50}'s and those given for the LD_{50}'s. The purities of compound nos. 3 and 7 are the greatest consistent with the results. It is possible that the values are too great by a factor of 2. The ratio of LD_{50}/I_{50} is not affected by these errors, as the LD_{50} and I_{50} for each was determined on the same solution assuming the same purity.

In finding the LD_{50} or I_{50} of a sulphonium compound what was actually found was the activity of an aqueous solution containing a known concentration of combined

phosphorus. If the activity could be attributed to compounds which did not contain phosphorus, then the results would be spurious. Three types of evidence showed that the results should be attributed to phosphorus anti-AChE's.

(1) Compound nos. 1, 2 and 5 have been studied in detail.[8] The inhibited AChE's had the same stabilities as those inhibited by other dimethyl and diethyl phosphorylated-AChE's, and the intensity and duration of symptoms was related to the degree of AChE inhibition *in vivo* and the rates of re-activation of the inhibited enzymes.

(2) All the compounds induced typical cholinergic symptoms in rats. The cationic compounds induced signs faster and of shorter duration than the uncharged compounds, but there were no other differences.

(3) The LD_{50}/I_{50} ratios for all the compounds are similar, suggesting that all act in the same way.

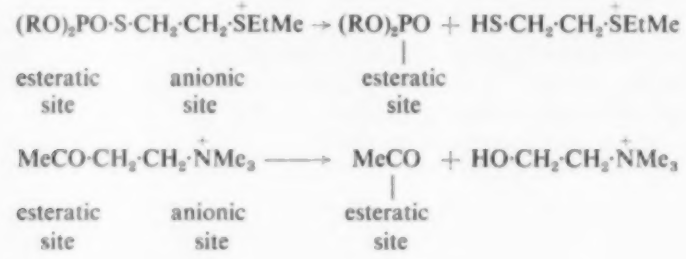
The sulphonium compounds were very much more toxic and biochemically active than the thioalkyl compounds from which they were derived. The high activity of the sulphonium compounds can be explained in the same way as the high activity of the phosphostigmines already referred to.[2] The cationic head is adsorbed on the anionic site of AChE, and greatly increases the probability that the esteratic site will be phosphorylated.

A check on this theory is available. If the rate of reaction with AChE is increased on forming a methylsulphonium derivative by a factor, f, and this factor arises solely from the stronger adsorption on the enzyme of the sulphonium compound, then the free energy of adsorption is given by $\Delta F = -RT \ln f$.[9] For the pair nos. 1 and 2, the factor is 1665, and for pair nos. 4 and 5 is 745. This gives $\Delta F = -4{\cdot}6$ kcal/mole for the first pair and $-4{\cdot}1$ kcal/mole for the second. The additional methyl group in each sulphonium compound probably contributes about $-1{\cdot}2$ kcal to these energies,[10] so the calculated energy of attraction of the positive charge on the sulphur for the anionic site is $-3{\cdot}5$ and $-2{\cdot}9$ kcal, respectively. A sulphonium ion has a pyramidal structure, and should therefore be able to approach the anionic site more closely than a quaternary nitrogen atom in, for example, acetylcholine. The energies calculated are consistent with this—in ACh the $\dot{N}$–anionic site energy is rather less, about 2·1 kcal.[9, 11]

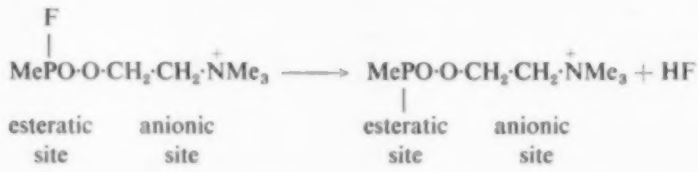
Consider now the pair nos. 8 and 9, in which the sulphur atoms are separated by a chain of three carbon atoms. For this pair $\Delta F = -2{\cdot}9$ kcal, a value not much less than for those compounds with two carbon atoms only in the chain, i.e. the increase in rate of reaction brought about by converting a compound to its methylsulphonium derivative does not vary much with chain length. The lengths of the chains measured from the central atoms in the ester group to the cationic group are 4·65 Å in ACh (carbonyl carbon to $\dot{N}$), 5·75 Å in the dimethylene compounds and 7·0 Å in the trimethylene compounds. (P to $\dot{S}$). The chain lengths were calculated from the bond-lengths tabulated by the Chemical Society.[12] It is obvious that the phosphorus compounds can only fit the enzyme by folding. Probably the greater flexibility of a trimethylene chain largely compensates for its greater length. This conclusion is independent of the observation that the trimethylene compounds are each less active than their dimethylene analogues, i.e. that compound no. 8 is less active than compound no. 4, and compound no. 9 than compound no. 5. Perhaps the ester bonding is more stable in the trimethylene compounds.

The only compounds of similar toxicity to some of the sulphonium compounds are some of the P:P-(fluoro)(methyl)phosphinylcholines.[1] The two groups are not as similar

as appears at first sight. Two of the sulphonium compounds have been shown to react with AChE to give dimethyl- and diethyl- phosphorylated AChE, respectively.[8] The first stage of the reaction has a marked resemblance to the first stage in the hydrolysis of ACh by AChE:



In both cases the group removed is the group adsorbed on the anionic site of the enzyme. The (fluoro)(methyl)phosphinylcholines appear to react differently:



Thus the group displaced by AChE is generally the group displaced by hydroxyl ions during alkaline hydrolysis, in these compounds the fluorine atom.[13] Also human AChE inhibited by such compounds is not re-activated *in vitro* by 2-hydroxyiminol methylpyridinium methiodide (P2-AM).[14] This result is only readily explained if the anionic site, on which P2-AM is adsorbed during re-activation[15] is shielded by a strongly adsorbed group. These considerations suggest that the adsorption of the quaternary nitrogen group on the anionic site of AChE may not contribute much to the anti-AChE activity of the (fluoro)(methyl)phosphinylcholines. It can only do so if the reaction at the esteratic site does not depend on the steric disposition of the groups. Otherwise if in ACh the adsorbed choline group is in a particularly favourable position for reaction to take place, in the phosphinylcholines the fluorine atom must be in an unfavourable position. There is some confirmation of this view. (Fluoro)-(methyl)phosphinylhomocholine is more biochemically active and toxic than its choline homologue.[1] In addition for many compounds rates of reaction with AChE are roughly correlated with rates of hydrolysis by water or by hydroxyl ions.[16] The phosphinylcholines are much less stable in water than several of the sulphonium compounds,[1, 17] so that the high activity of fluorophosphinyl compounds may be due to the readiness with which their P—F bonds are broken.

Five of the sulphonium compounds (nos. 3, 5, 6, 7 and 10) are more toxic than any non-ionic phosphorus compounds on which there are reports. The only other phosphorus compounds of comparable toxicity are also cationic. Thus the most toxic compounds are all ionic, and do not penetrate the central nervous system.[1, 2, 8] It is

obvious that action on the central nervous system is not necessary for death to ensue from cholinergic effects.

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SOME EFFECTS OF RESERPINE ON BARBITONE ANAESTHESIA IN MICE

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Abstract—Barbitone induction time has been measured at various intervals after pretreating mice with reserpine: special attention was paid to the rate of penetration of the barbitone into the brain. The period of sedation resulting from intraperitoneal administration of reserpine coincides with a period in which barbitone induction time is greatly reduced. Two days after reserpine the mice are no longer sedated, and barbitone induction time is normal. From 3 to 4 days after reserpine the animals are hyperexcitable, and this phase coincides with one in which barbitone induction time is greatly increased. The mice become normal for the second time from 5 to 6 days after reserpine. It is shown that the increased induction time results from a slower rate of penetration of barbitone into the brain.

INTRODUCTION

Beiler, *et al.*[1] have reported that certain enzymes reduce the induction time of barbitone in mice, but do not influence the duration of hypnosis by hexobarbitone. They concluded that the enzymes increased the permeability of the blood–brain barrier to barbitone, thus hastening the onset of hypnosis. We have investigated the effects of other substances, especially those with pronounced central action, on the induction time of barbitone and its rate of penetration into the brain, and have shown that a reduction in this time does not necessarily imply an increase in rate of penetration of barbitone into the brain.[2] During these experiments it was observed that the initial potentiation (reduced induction time) produced by reserpine reverted to antagonism (increased induction time) a few days later. Previously we had noticed a similar reversal in the effect of reserpine on the duration of hexobarbitone-induced hypnosis.[3] It was observed also that mice were easily excited after recovering from the sedation induced by reserpine. The experiments described below were carried out in an attempt to correlate post-reserpine hyperexcitability with the reduction observed in barbitone activity.

METHODS

Male fawn mice (GFF strain, from 16 to 22 g) were used in all the experiments.

The induction time of barbitone hypnosis was measured by the method of Greig and Mayberry.[4] Groups of mice were injected intravenously with sodium barbitone (5·5 mg in 0·4 ml/20 g), each injection taking from 20 to 30 sec. The interval between the injection and the loss of righting reflex was recorded as the induction time. The experiments were conducted at room temperature (approximately 19 °C).

Groups of mice given barbitone only were included in every experiment involving measurements of induction time.

Barbitone concentrations in the brain were determined by Butler's method.[5] Groups of four mice were injected intravenously with sodium barbitone (5·5 mg in 0·4 ml/20 g) and then killed after 2, 5, 10, 15, 20, 25, 30 or 35 min. The brains from each group were weighed together and assayed for total barbitone.

TABLE 1. CHANGES IN BARBITONE INDUCTION TIME PRODUCED BY RESERPINE OR CHLORPROMAZINE

Time after injecting drug	Group* mean induction time (min ± s.e.)			
	After reserpine (2 mg/kg i.p.)		After chlorpromazine (2 mg/kg i.p.)	
	Test	Control	Test	Control
1 hr	8·0 ± 0·4	19·5 ± 1·0	11·3 ± 0·6	20·0 ± 0·9
2 hr	4·9 ± 0·2	19·5 ± 1·0	not tested	
3 hr	3·2 ± 0·3	19·5 ± 1·0	10·7 ± 0·8	20·0 ± 0·9
6 hr	3·8 ± 0·4	20·0 ± 1·0	15·9 ± 0·3	20·0 ± 0·9
1 day	12·9 ± 1·3	19·2 ± 0·6	18·9 ± 0·7	21·1 ± 0·6
2 days	20·3 ± 0·7	18·3 ± 0·7	19·4 ± 0·8	18·6 ± 0·5
3 days	34·5 ± 1·2	17·7 ± 0·5	21·2 ± 0·7	19·0 ± 0·6
4 days	31·3 ± 2·8	18·1 ± 1·3	20·3 ± 0·9	20·4 ± 0·8
5 days	22·6 ± 0·9	18·3 ± 0·9	20·8 ± 0·5	20·5 ± 0·6
6 days	20·2 ± 0·8	19·0 ± 0·4	21·0 ± 0·7	20·1 ± 0·7
7 days	19·0 ± 1·1	18·0 ± 1·3	21·0 ± 0·8	21·0 ± 0·7

* Ten animals per group.

TABLE 2. BRAIN BARBITONE CONCENTRATIONS AFTER PRETREATMENT WITH RESERPINE

Time after intraperitoneal reserpine (2 mg/kg)	Brain barbitone concentration (moles × 10^{-6}/g wet tissue)*								Brain barbitone concentration at induction (moles × 10^{-6}/g wet tissue)
	Time in minutes after intravenous injection of sodium barbital (275 mg/kg)								
	2	5	10	15	20	25	30	35	
1 hr	0·38	0·59	0·74	0·86	0·93	1·03	1·06	1·06	0·69
2 hr	0·35	0·57	0·82	0·84	0·93	1·02	1·00	1·07	0·58
1 day	0·37	0·54	0·86	1·03	1·06	1·12	1·07	1·12	1·00
2 days	0·38	0·61	0·79	0·95	1·04	1·14	1·00	1·07	1·08
3 days	0·55	0·64	0·82	0·97	1·02	1·04	1·07	1·12	1·08
4 days	0·48	0·65	1·00	1·03	1·08	1·15	1·12	1·18	1·16
5 days	0·49	0·64	0·91	1·00	1·08	1·14	1·12	1·20	1·13
6 days	0·51	0·71	0·93	1·09	1·17	1·14	1·24	1·26	1·18
7 days	0·49	0·68	0·97	1·04	1·10	1·07	1·12	1·12	1·09
Controls, untreated	0·52	0·71	0·89	1·05	1·17	1·17	1·24	1·18	1·12

* Each value is the mean of two results.

RESULTS

The changes in barbital induction time produced by pretreating the mice with reserpine (2 mg/kg intraperitoneally) are given in Table 1, and the changes in the rate of penetration of barbitone into the brain in Table 2.

Induction time was shortest from 3 to 6 hr after administering reserpine. It returned to normal after approximately 2 days, but during the third and fourth day the induction time was markedly increased. Finally, after about 6 days, the induction time returned to normal for the second time.

The rate of penetration of barbitone into the brain was reduced by pretreatment with reserpine, and during the first day the barbitone concentration in the brain at the onset of hypnosis was appreciably less than in control animals. The curves for barbitone concentration in the brain plotted against time for the control mice and for those receiving reserpine three days before the barbitone are shown in Fig. 1. The points

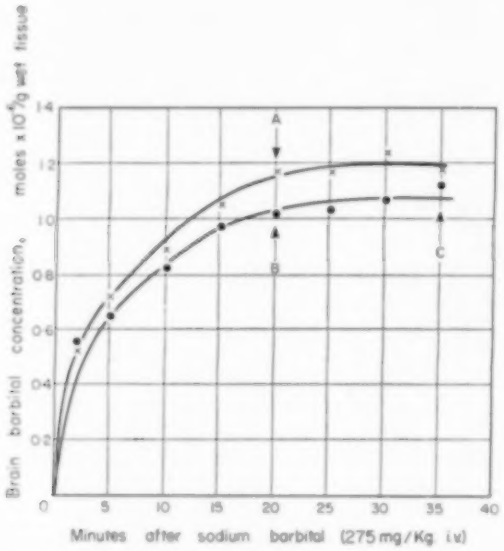

Fig. 1. The effect of reserpine on the rate of penetration of sodium barbitone into mouse brain: ×——× barbital alone; ●——● barbital 3 days after intraperitoneal reserpine 2 mg/kg.

marked *A*, *B* and *C* represent the barbitone concentrations in control mice at induction (*A*), in reserpinized mice at the induction time of normal mice (*B*) and in reserpinized mice at induction (*C*). We wished to know whether the concentrations at *A*, *B* and *C* were in fact significantly different from one another; hence a further eighty mice received an intraperitoneal injection of reserpine (2 mg/kg) and sodium barbitone intravenously 3 days later. Ten groups of four mice were killed 20 min after the barbitone (*B*) and the other ten groups 35 min after (*C*). Ten groups of four mice that had not received reserpine were killed 20 min after intravenous barbitone (*A*) and the concentrations of barbitone in their brains were measured. The results are given in Table 3, together with a Student *t* test for significance of difference between the groups. It will be seen that the brain barbitone concentration at *B* differs significantly from those at both *A* and *C* ($P < 0{\cdot}01$), whereas the latter two values do not differ from one another ($P < 0{\cdot}2$). Thus the increase in induction time 3 days after reserpine is simply a reflection of the reduced rate of entry of barbitone into the brain, the concentration at induction remaining unaltered.

The results of the above experiments prompted us to study the effects of chlorpromazine under similar conditions. Chlorpromazine (2 mg/kg intraperitoneally) reduced barbitone induction time, the reduction being greatest from 1 to 3 hr after dosing. The induction time returned to normal within 2 days and remained unchanged during the next 5 days (Table 1).

TABLE 3. BRAIN BARBITONE CONCENTRATIONS AT POINTS A, B AND C IN FIG. 1

Brain barbitone concentrations (moles × 10^{-6}/g wet tissue)		
Controls, no reserpine	Tests, 3 days after reserpine (2 mg/kg i.p.)	
Point *A* 20 min after sodium barbitone i.v.	Point *B* 20 min after sodium barbitone i.v.	Point *C* 35 min after sodium barbitone i.v.
1·12	1·04	1·11
1·09	1·01	1·13
1·18	1·04	1·20
1·06	1·06	1·13
0·98	1·04	1·11
1·06	0·97	1·11
1·10	1·03	1·10
1·10	1·06	1·20
1·16	0·98	1·15
1·16	1·05	1·06
1·10 ± 0·02*	1·03 ± 0·01*	1·13 ± 0·01*

* Standard error of mean.

Student '*t*' between *A* and *B* = 3·44 ($P < 0·01$).

Student '*t*' between *A* and *C* = 1·25 ($P > 0·2$).

Student '*t*' between *B* and *C* = 5·98 ($P < 0·001$).

DISCUSSION

The experiments with reserpine described above show that the period of sedation and barbitone potentiation (reduced induction time) gives place to one of excitation and barbitone antagonism (prolonged induction time). The finding of excitation agrees basically with that of Taeschler and Cerletti,[6] who showed that the stimulant effects of lysergic acid diethylamide in mice are potentiated by pretreatment with reserpine, and that of Frommel and Gold,[7] who reported that guinea-pigs are excitable and difficult to handle a few days after receiving the total alkaloids of Rauwolfia.

It is interesting to recall the suggestion of Lessin and Parkes,[8] that the sedative and pentobarbitone-potentiating actions of reserpine in mice kept at room temperature are related to the drug's hypothermic action. More recently, these workers[9] have shown that the duration of reserpine's sedative and hypothermic actions differs from that for its facilitation of leptazole convulsions, the time-course of the latter following more closely that of the reduction in the concentration of brain 5-hydroxytryptamine.

Our own experiments show that the action of reserpine on the rate of entry of barbitone into the brain is one of consistent reduction, the time course also following closely that of the reduction of mouse brain 5-HT.[10] It would have therefore been reasonable to expect that the increased induction time experienced 3 and 4 days after

reserpine should also occur during the first 2 days. Since this is not so, we believe that the action of barbitone is impeded by reserpine during the first 2 days, but this inhibition is masked by an overwhelming potentiation, possibly resulting from hypothermia.

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CONTRASTING EFFECTS OF THYROXIN ON ZOXAZOLAMINE AND HEXOBARBITAL METABOLISM

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Abstract—Administration of thyroxin to rats: (a) shortens the duration of action of zoxazolamine (2-amino-5-chlorobenzoxazole by accelerating its metabolism, and (b) prolongs the duration of action of hexobarbital by inhibiting its metabolism. Administration of the hormone does not alter the activity of the zoxazolamine-metabolizing enzyme system in liver microsomes, but does decrease the activity of the hexobarbital-metabolizing enzyme system in liver microsomes. Restricting the diet of the rats, so that their gain in weight was the same as the rats treated with thyroxin, inhibits the metabolism of hexobarbital, but does not alter the metabolism of zoxazolamine.

PRETREATMENT of rats with certain drugs increases the activity of microsomal enzymes in liver that metabolize the same drug or another drug.[1] This adaptive response to drug administration leads to a shortened duration of drug action. Because of reports that patients with thyrotoxicosis differ from normal individuals in their response to various drugs[2–4] and because of the observation of McGuire and Tomkins[5] that pretreatment of rats with thyroxin increases Δ^4-3-keto-steroid hydrogenases in liver microsomes, we have investigated the effects of pretreatment of rats with thyroxin on the metabolism of drugs.

METHODS

Animals

Male Sprague–Dawley rats weighing 70–80 g were fed Purina Laboratory Chow. They were injected daily with 0·2 mg of sodium l-thyroxin intraperitoneally. Sodium thyroxin was dissolved in a dilute solution of sodium hydroxide which was then neutralized with dilute hydrochloric acid. The fine flocculent precipitate of thyroxin formed was dispersed by shaking before injections were given. Control rats received injections of physiological saline. Solutions of zoxazolamine and hexobarbital were prepared as previously described.[1] Since the thyroxin-treated rats did not gain weight as rapidly as the controls, three groups of animals were used: (a) controls fed *ad libitum*, (b) controls fed a restricted diet and (c) thyroxin-treated fed *ad libitum*. The weights of the control *ad libitum*-fed rats at 18–20 days after the start of the experiment ranged from 150–170 g, while the weights of rats in the other two groups were 105–130 g. Thyroxin was administered for 18–20 days in all experiments except for the experiment described in Fig. 2.

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Drug metabolism studies

The rats were killed by decapitation for *in vitro*-enzyme assays. The livers were removed and homogenized at 0–5 °C in 2 vols. of 0·25 M sucrose solution. Liver microsomes were prepared and were assayed for enzyme activity as previously described.[1] Microsomes from 200 mg of liver were incubated with 0·67 μmole of zoxazolamine, while microsomes from 660 mg of liver were incubated with 1·0 μmole of hexobarbital. The drugs were incubated by shaking in air for 30 min at 37 °C with liver microsomes in the presence of a system that generated reduced TPN (TPNH) and the metabolism of zoxazolamine and hexobarbital was determined by measuring the disappearance of these drugs.[1]

For studies on the *in vivo*-metabolism of drugs, rats were injected intraperitoneally with 100 mg of zoxazolamine per kg or with 125 mg of hexobarbital per kg. The animals were killed with chloroform and were immediately skinned and homogenized in 2 vols. of water in a Waring Blendor. An aliquot of the homogenized rat was analysed for drug.[1, 6]

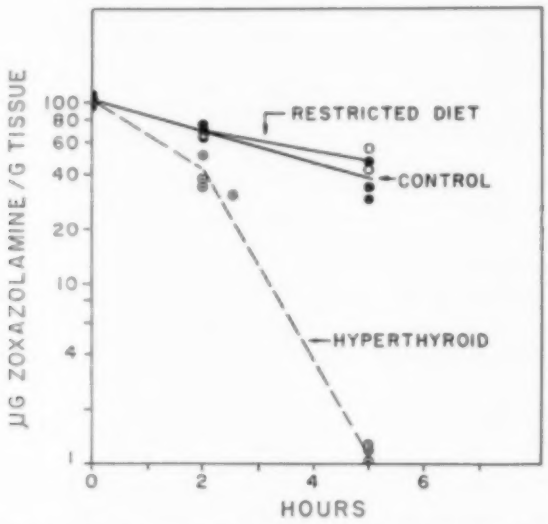

FIG. 1. The ability of thyroxin to accelerate the metabolism of zoxazolamine *in vivo*. The rats were injected intraperitoneally with 100 mg of zoxazolamine per kg and were killed at intervals. The remaining drug in the rat was estimated as described in the Methods section. Each point represents the value obtained from a single rat.

Assay for glucose-6-phosphate and 6-phosphogluconate dehydrogenase activity

Livers were homogenized in 9 vols. of 0·25 M sucrose solution. The homogenate was centrifuged at 100,000 *g* for 60 min and the supernatant fraction was assayed for glucose-6-phosphate and 6-phosphogluconate dehydrogenase as described by Huggins.[7]

EXPERIMENTAL

Effect of thyroxin on the metabolism of zoxazolamine

Pretreatment of rats with thyroxin shortened the duration of zoxazolamine paralysis from 770 min to 170 min (Table 1). To investigate the possibility that thyroxin

shortened the action of zoxazolamine by accelerating its metabolism, the rate of metabolism of this drug *in vivo* was studied in control and hyperthyroid rats. Fig. 1 shows that zoxazolamine was metabolized more rapidly *in vivo* by hyperthyroid rats than by control rats. An additional experiment showed that control or hyperthyroid rats that were injected intraperitoneally with 100 mg of zoxazolamine per kg did not excrete this drug in their urine.

Previous experiments have shown that pretreatment of rats with various foreign compounds such as phenobarbital and 3:4-benzpyrene accelerated zoxazolamine

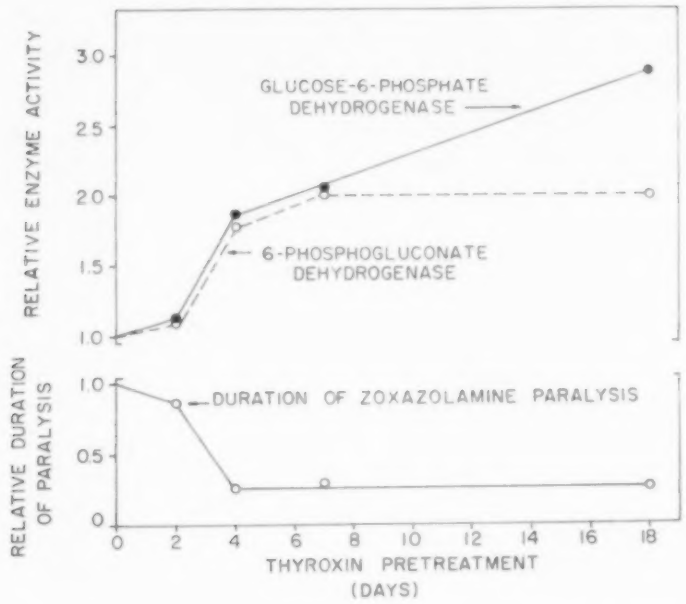

FIG. 2. The ability of thyroxin to shorten the duration of paralysis caused by zoxazolamine and to increase the activity of enzymes that generate reduced TPN. Rats were given daily intraperitoneal injections of 0·2 mg of sodium thyroxin. The duration of paralysis induced by zoxazolamine in control and thyroxin-treated rats was determined as described in the footnote to Table 1. From seven to ten rats were used per group. The data are expressed in relative units, with the controls taken as 1·0. Other rats were injected with thyroxin or saline. Their livers were homogenized and were centrifuged at 100,000 *g* for 60 min. The supernatant fractions were assayed for glucose-6-phosphate and 6-phosphogluconate dehydrogenase. Four rats were used per group and the enzyme activities per g of liver are expressed in relative units, with the controls taken as 1·0.

metabolism by increasing the activity of the enzyme system in liver microsomes which metabolizes zoxazolamine. It was expected that thyroxin might act in a similar manner. However, pretreatment of rats with thyroxin did not significantly increase the activity of this enzyme system (Table 1). In these experiments the liver microsomes were isolated and were fortified with a system that generated excess reduced TPN so that the microsomes were limiting for the metabolism of zoxazolamine. Liver microsome from control rats metabolized 0·92 μmole zoxazolamine per g of liver per hr, microsomes from the rats on the restricted diet metabolized 0·83 μmole and the

microsomes from thyroxin-treated rats metabolized 1·0 μmole. Similar rates of zoxazolamine metabolism were obtained when whole liver homogenate or the supernatant fraction obtained by centrifuging homogenate at 9000 *g* for 15 min were assayed for ability to metabolize this drug in the presence of excess TPN. Attempts to show differences in the rate of zoxazolamine metabolism by unfortified liver homogenates or by liver slices failed because insufficient drug was metabolized to allow an accurate estimation of its metabolism. The possibility was investigated that thyroxin might increase the activity of a zoxazolamine-metabolizing enzyme system in some tissue other than liver. Fortified homogenates of kidney, spleen, intestine, testis or muscle obtained from thyroxin-treated rats, however, did not metabolize zoxazolamine.

An interesting property of thyroid hormone is its ability to stimulate liver growth and synthesis of liver protein.[8, 9] In our experiments the liver weight (g per 100 g body weight) for the control, restricted diet, and thyroxin-treated (18 days) groups were 4·42 ± 0·24, 4·47 ± 0·28 and 5·38 ± 0·22, respectively. Ten rats per group were

Table 1. Effect of thyroxin administration on duration of zoxazolamine paralysis and on zoxazolamine metabolism by liver microsomes

Group	Zoxazolamine paralysis (min)		*In vitro* metabolism (μmoles metabolized/g liver/hr)
Control	770 ± 214	(6)	0·92 ± 0·07
Restricted diet	895 ± 82	(6)	0·83 ± 0·10
Hyperthyroid	170 ± 43	(9)	1·01 ± 0·08

The duration of drug action was determined by measuring when the animals regained their righting reflex after an intraperitoneal injection of 163 mg of zoxazolamine per kg. The average value and the standard deviations are given. The number of rats used in each experiment is included in parentheses.

Rat liver microsomes were assayed for ability to metabolize zoxazolamine, as described in the Methods section. The average and standard deviation of seven experiments are given. Pooled livers from three to four animals were used for each experiment.

used in this study and the standard deviations are given. When the *in vitro*-data on the metabolism of zoxazolamine, shown in Table 1, are expressed as μmoles metabolized per 100-g rat, the extent of zoxazolamine metabolism by microsomes from the hyperthyroid group is 34 and 43 per cent greater than the control and restricted diet groups, respectively. Although the increased liver:body weight ratio observed in rats treated with thyroxin can partially explain the accelerated metabolism of zoxazolamine observed *in vivo*, it is unlikely that the large effect of the hormone on the metabolism of zoxazolamine can be entirely explained by such a mechanism.

Previous studies have shown that pretreatment of rats with thyroxin increases the activities (per g of liver) of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, enzymes involved in the generation of reduced TPN.[10] It seemed possible that thyroxin may accelerate zoxazolamine metabolism *in vivo* by increasing the supply of available reduced TPN, a cofactor that is necessary for the metabolism of zoxazolamine. In order to test this possibility, thyroxin was injected daily and an attempt was made to correlate the shortened duration of action of zoxazolamine with the rise in enzymes that generate reduced TPN. Fig. 2 shows that the duration of action of zoxazolamine was shortened at the time that the activities

of 6-phosphogluconate and glucose-6-phosphate dehydrogenases were elevated. Although these experiments suggest that the levels of enzymes that generate reduced TPN may influence zoxazolamine metabolism, definite conclusions on this point must await further studies.

Effect of thyroxin on the metabolism of hexobarbital

In contrast to the results obtained with zoxazolamine, pretreatment of rats with thyroxin prolonged the duration of action of hexobarbital (Table 2). The explanation for this came from finding that thyroxin administration markedly decreased the

TABLE 2. EFFECT OF THYROXIN ADMINISTRATION ON DURATION OF HEXOBARBITAL HYPNOSIS AND ON HEXOBARBITAL METABOLISM BY LIVER MICROSOMES

Group	Hexobarbital-sleeping time (min)		*In vitro* metabolism (μmoles metabolized/g liver/hr)
Control	28 ± 4	(10)	1·47 ± 0·09
Restricted diet	69 ± 23	(10)	0·90 ± 0·14
Hyperthyroid	81 ± 65	(10)	0·79 ± 0·13

The sleeping time was determined by measuring when the animals regained their righting reflex after an intraperitoneal injection of 125 mg of hexobarbital per kg. The average value and the standard deviations are given. The number of rats used in each experiment is included in parentheses.

Rat liver microsomes were assayed for ability to metabolize hexobarbital, as described in the Methods section. The average and standard deviation of four experiments are given. Pooled livers from three or four animals were used for each experiment.

TABLE 3. EFFECT OF THYROXIN ADMINISTRATION ON THE METABOLISM OF HEXOBARBITAL *in vivo*

Group	μg Hexobarbital/g tissue			
	30 min		60 min	
Control	*35*	(35, 22, 47)	*21*	(15, 24, 25)
Restricted diet	*58*	(58, 58, 59)	*35*	(30, 40)
Hyperthyroid	*51*	(63, 47, 44)	*36*	(34, 39, 35)

The rats were injected intraperitoneally with 125 mg of hexobarbital per kg and were killed 30 min and 60 min later. The amount of drug remaining in the animal was estimated as described in the Methods sections. The average amount of hexobarbital per g of tissue is italicized and is followed by the individual values in parentheses.

activity of the hexobarbital-metabolizing enzyme system in liver microsomes (Table 2). In these experiments, the rats on a restricted diet also had a prolonged sleeping time and decreased activity of the hexobarbital-metabolizing enzyme system. These effects of thyroxin and of a restricted diet to inhibit *in vitro*-metabolism of hexobarbital were paralleled *in vivo* by decreased rates of hexobarbital metabolism (Table 3). Although rats treated with thyroxin eat greater quantities of food than *ad libitum*-fed control rats, they do not gain weight as rapidly as the controls. The possibility should be

considered that thyroxin may inhibit the hexobarbital-metabolizing enzyme system by producing a condition which in certain respects resembles starvation. The results observed here with a restricted diet are in accord with the results of Dixon *et al.*[11] who found decreased levels of this enzyme system in starved mice.

DISCUSSION

Pretreatment of rats with thyroxin shortens the duration of action of a subsequent injection of zoxazolamine by accelerating its metabolism. Thyroxin does not appear to act like various foreign compounds such as phenobarbital and 3:4-benzpyrene which accelerate zoxazolamine metabolism *in vivo* by markedly increasing the activity per g of liver of the zoxazolamine-metabolizing enzyme system in liver microsomes.[1] The activity of the zoxazolamine-metabolizing enzyme system per g of liver is not increased by thyroxin administration. The effect of the hormone on zoxazolamine metabolism can be partially explained by its ability to increase the liver to body weight ratio. This increased ratio causes about a 34 per cent increase in the activity of the zoxazolamine-metabolizing enzyme system per 100-g rat. The possibility has also been pointed out here that thyroxin may accelerate *in vivo*-metabolism of zoxazolamine, in part by increasing the activity of liver enzymes that generate reduced TPN. The ability of thyroxin to accelerate the metabolism of steroids by increasing the activity of liver enzymes that generate reduced TPN has been demonstrated by McGuire and Tomkins.[5]

In contrast to the results obtained with zoxazolamine, pretreatment of rats with thyroxin prolonged the duration of action of hexobarbital by decreasing the activity of the hexobarbital-metabolizing enzyme system in liver microsomes. The finding that thyroxin decreased the activity of the enzyme system which metabolizes hexobarbital is similar to the results of Cochin and Sokoloff,[12] who found that administration of thyroxin decreased the activity of the enzyme system in liver microsomes which N-demethylates morphine.

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EVALUATION OF SOME UNCOUPLING AGENTS IN THE CHEMOTHERAPY OF EXPERIMENTAL CANCER: A STUDY OF THEIR DISTRIBUTION IN TUMOUR-BEARING RATS*

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Abstract—Four nitrophenol compounds known as uncoupling agents were administered to tumour-bearing rats to study the distribution in the body.

It was not possible to attain the concentration in the tumour, that was assumed on the basis of experiments *in vitro* to be fatal for the tumour, without endangering the life of the animal.

The half-clearance time of the compounds was in good agreement with the lipid solubility and the protein-binding capacity.

INTRODUCTION

CLOWES and Krahl[1] showed that 2:4-dinitrophenol and related compounds interfere with cell division. Much later it was found that a fundamental action of these compounds consists of the uncoupling of oxidative phosphorylation,[2] with the result that the affected cell can no longer utilize the respiratory reaction chain for building up "energy-rich" phosphates.

The rapid proliferation of tumour tissue and the consequent high energy requirement for cell division induced us to determine whether systemic administration might inhibit the proliferation of tumour tissue more markedly than that of normal tissue.

These considerations led us first to determine for a small series of uncoupling agents the concentration required *in vitro* for uncoupling of oxidative phosphorylation in cancer cells.†

The second stage of our experiments consisted of attempts to achieve this effective concentration in experimental tumours *in vivo* by systemic administration of these uncoupling agents in doses that would be tolerated satisfactorily by the animal.

While this aim was not achieved the experiments gave some information on the distribution of these uncoupling agents in tumour-bearing rats.

Dinitrophenol, *p*-nitrophenol, dinitrocarvacrol and dinitrothymol were selected as the uncoupling agents to be used in this work. The two latter compounds were chosen

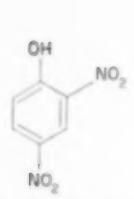
* This work was initiated by Professor Bruno Mendel, F.R.S., and carried out under his direction until his death on 23 August 1959. The paper has been prepared by his co-workers to commemorate this gifted investigator.

† Further experiments with the same uncoupling agents in these concentrations *in vitro* indicated that under certain conditions these compounds may indeed destroy the viability of tumour cells (unpublished experiments).

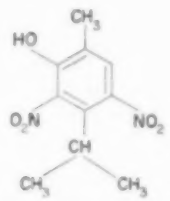
from the series of compounds studied by Clowes and Krahl[1]: dinitrocarvacrol because of its high activity, and dinitrothymol because unlike many other uncoupling agents it does not stimulate cell respiration.

MATERIAL AND METHODS

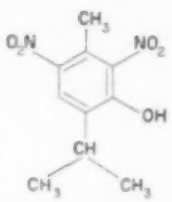
Dinitrophenol and *p*-nitrophenol were obtained commercially and purified by crystallization; the butyric ester of *p*-nitrophenol was prepared from *p*-nitrophenol and butyrylchloride in the usual way and purified by distillation. Dinitrocarvacrol and dinitrothymol were synthesized in this laboratory following a modified method of Mazzara[3]. Their formulae together with the abbreviations by which they will be referred to in this paper are shown below:



2:4-dinitrophenol (DNP)



3:5-dinitrocarvacrol (DNCa)



2:6-dinitrothymol (DNT)

p-nitrophenol (PNP)

Arachis oil or 0·21 per cent (w/v) $NaHCO_3$ was used as a vehicle for the subcutaneous administration; the latter was used when intravenous injection was required.

DNP and PNP concentrations in plasma and tissue homogenates were determined by the method of Mudge and Taggart[4]. This technique was also used for the determination of DNCa and DNT in plasma, but proved to be unsuitable for the determination of these two compounds in tumour, liver and brain homogenate. Attempts to improve the results of recovery experiments by using different organic solvents or Zander and Simmer's[5] extraction method were unsuccessful. The following method was eventually adopted. A tissue homogenate was divided into two equal portions, and 100 μg of DNCa (or DNT) was added to one. Both portions were acidified with 0·5 ml of 3 N H_2SO_4 and extracted with 25 ml of ether. An aliquot of 20 ml of each extract was shaken with 5 ml of 0·05 M borax–NaOH buffer solution of pH 10·6, and the absorbancy of the buffer layer measured in a spectrophotometer at a wavelength of 400 mμ. The percentage recovery of the 100 μg of DNCa (or DNT) added to the homogenate was calculated from the difference between the absorbancies of the two extracts obtained, and it was assumed that the same percentage of DNCa (or DNT) had been extracted from the homogenate to which no DNCa (or DNT) was added before extraction.

Swiss mice inoculated with the Ehrlich mouse-ascites carcinoma* were used for obtaining ascites fluid and tumour cells for the experiments *in vitro*. Adult male rats from the strain bred in this institute (an inbred crossing of Piebald and Wistar rats) were used to study the absorption and elimination of the uncoupling agents. Adult

* We are indebted to Dr. G. Klein, Stockholm, for supplying us with the Ehrlich ascites carcinoma, and to Professor O. Mühlbock, Amsterdam, for supplying us with the rat mammary tumour DE 40.

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male Wistar rats with the mammary tumour DE 40* were used in the experiments on the distribution of the uncoupling agents in tumour-bearing rats.

Uncoupling of oxidative phosphorylation *in vitro* was studied by measuring the effect on aerobic glycolysis. For comparison between the different uncoupling compounds, the concentrations required to bring the aerobic glycolysis up to the level of the anaerobic were compared.

The aerobic and anaerobic glycolysis of the Ehrlich mouse-ascites carcinoma were measured manometrically by Warburg's method[6] in a medium of either cell-free ascites fluid or Krebs–Ringer–bicarbonate solution,[6] to which glucose was added to a concentration of 180 mg/100 ml and the uncoupling agent in varying concentrations. The ascites fluid was used to test the extent to which binding of the uncoupling agent by protein would give protection *in vivo*. Standard volumes of 0·2 ml of cell suspension (60–80 mm^3 cells) and 5·0 ml of medium were employed throughout the experiments.

The affinity of the uncoupling agents for protein was evaluated by filtering a solution of each compound in ascites fluid through cellophane under a pressure of 15 cm of mercury, and measuring the concentration of uncoupling agent in the protein-free filtrate.

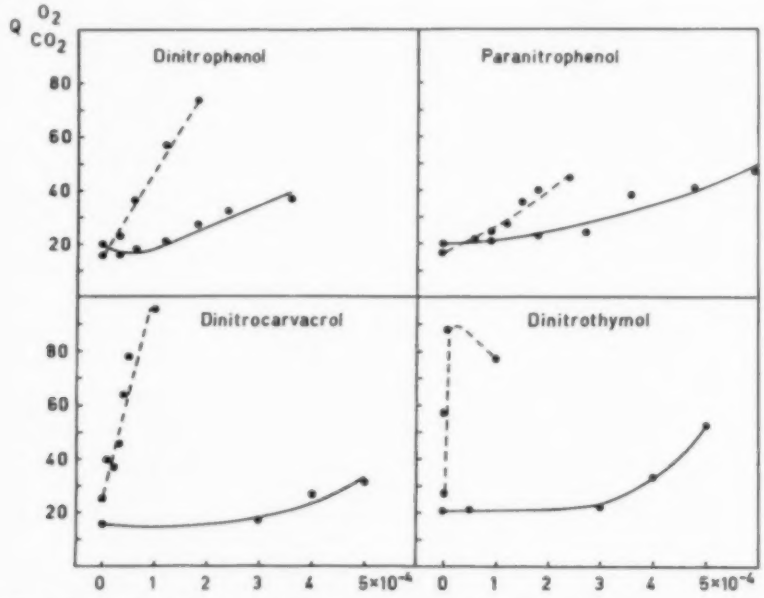

FIG. 1. The effect of four uncoupling agents on the aerobic glycolysis of Ehrlich mouse-ascites carcinoma cells *in vitro*. The molar concentration is represented along the abscissa, the rate of glycolysis along the ordinate ($Q_{CO_2}^{O_2}$;). ●– –●: aerobic glycolysis in Krebs–Ringer–bicarbonate solution. ●——●: aerobic glycolysis in cell-free ascites fluid.

RESULTS

Fig. 1 shows the effect of the four uncoupling agents on the aerobic glycolysis of Ehrlich ascites-carcinoma cells *in vitro*, when these cells were suspended either in a Krebs–Ringer–bicarbonate solution or in cell-free ascites fluid. For the experiments *in vivo*, it was decided arbitrarily to aim at a concentration of uncoupling agent which

would bring the aerobic glycolysis up to the level of the anaerobic glycolysis in the absence of uncoupling agent, which amounted to doubling the aerobic glycolysis under our conditions. Fig. 1 shows that, in the presence of ascites fluid, this requires $3{\cdot}6 \times 10^{-4}$ M DNP, $4{\cdot}7 \times 10^{-4}$ M PNP, $4{\cdot}8 \times 10^{-4}$ M DNCa, or $4{\cdot}5 \times 10^{-4}$ M DNT.

It is clear that in the absence of ascites fluid the uncoupling agents are able to increase the aerobic glycolysis to a value considerably above that of the anaerobic glycolysis in the absence of uncoupling agent. This is in agreement with the observations of Emmelot and Bos[7] with the same tumour. These authors showed, moreover, that 10^{-4} M DNP more than doubled the rate of anaerobic glycolysis. The degree of stimulation of the aerobic glycolysis by 10^{-4} M DNP, reported by Emmelot and Bos, agrees well with the values shown in Fig. 1.

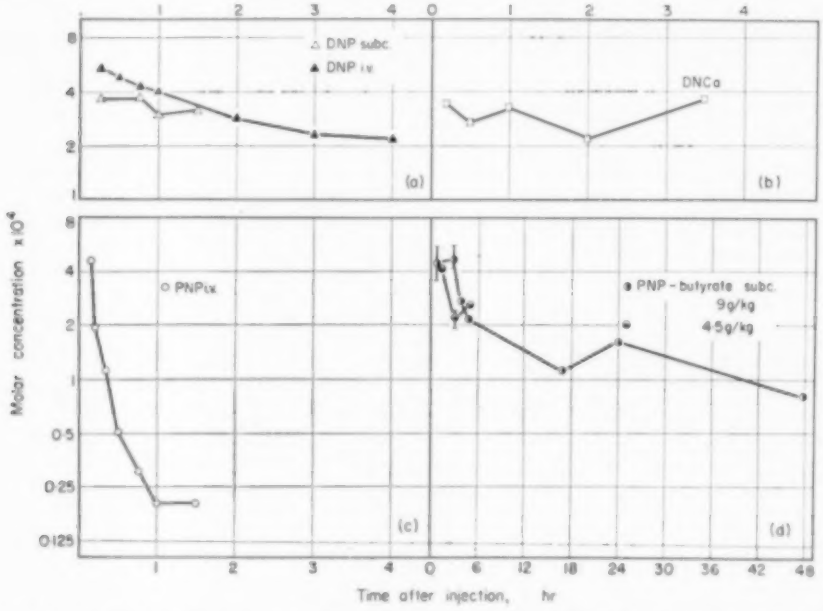

FIG. 2. The plasma level of dinitrophenol (DNP), *p*-nitrophenol (PNP) and dinitrocarvacrol (DNCa) in adult rats after parenteral administration of one dose.

(A). Individual rats were injected subcutaneously or intravenously with 20 mg DNP/kg body weight and killed at different intervals to determine the DNP concentration in the plasma. △——△ subcutaneously; ▲——▲: intravenously. (B). A similar experiment with 15 mg DNCa/kg injected intravenously. (C). The PNP concentration was studied in the same way after the intravenous administration of 75 mg PNP/kg. (D). PNP-butyrate was injected subcutaneously in a dose of 4·5(◒) or 9 g (◗)/kg, and the PNP concentration in the plasma was studied at the indicated intervals. When the concentration in the plasma was determined in two or more animals killed at the same time after injection the mean and range are indicated.

Normal rats of the same weight as those bearing tumours to be used later were injected either subcutaneously or intravenously with the uncoupling agents, and the concentration in the plasma was determined at various times after the injection, to find a dosage schedule that would yield at least the above concentrations for a reasonable time.

Tainter and Cutting[8] reported a value of 25 mg/kg for the LD_{50} of DNP for the rat on subcutaneous administration, while Spencer[9] found 30 mg/kg to be the LD_{50} for orally administered DNP. Therefore we chose a dose of 20 mg/kg, which was well tolerated both after subcutaneous and intravenous injection. As shown in Fig. 2 the desired minimum concentration of $3{\cdot}6 \times 10^{-4}$ M was maintained for at least 1·5 hr after a single intravenous injection of 20 mg/kg, while the half clearance is approximately 2 hr. In a subsequent experiment a male Wistar rat received an intravenous injection of 20 mg/kg, followed by four maintenance doses of 3·5 mg/kg 1, 2, 3 and 4 hr later. When the rat was sacrificed 5 hr after the initial injection a concentration of $3{\cdot}2 \times 10^{-4}$ M DNP was found in the plasma, indicating that the desired threshold concentration had been present for between 4 and 5 hr. However, when rats with the mammary tumour were treated according to this schedule the DNP concentration in the tumour itself never reached the threshold concentration, although the concentration in the plasma was in agreement with the corresponding findings in the preceding experiments.

In a pilot experiment with PNP two male Wistar rats received 100 mg/kg subcutaneously; after 25 min a PNP concentration of 3×10^{-5} M was found in the plasma of one of the rats, while 60 min after the injection no PNP could be found in the plasma of the other animal. Fig. 2 shows the results of a second experiment, in which seven rats were given 75 mg PNP/kg body weight intravenously, and were killed at short intervals for the determination of the PNP level in the plasma. These results indicated

TABLE 1. THE *p*-NITROPHENOL (PNP) CONCENTRATION IN THE PLASMA AND IN THE TUMOUR OF RATS WITH A MAMMARY TUMOUR DURING CONTINUOUS ADMINISTRATION OF THIS COMPOUND

(Three adult male Wistar rats were anaesthetized with sodium pentobarbitone (35 mg/kg), injected subcutaneously with 100 mg PNP/kg, and immediately after connected with a motor-driven syringe delivering 180 mg PNP/kg per hr subcutaneously. The PNP was dissolved in 0·21 per cent (w/v) aq.$NaHCO_3$.)

Rat no.	Interval between first injection and determination (min)	Concentration of PNP (M)		Fate
		In plasma	In tumour	
1	55	$4{\cdot}3 \times 10^{-4}$	$3{\cdot}7 \times 10^{-4}$	†
2	120		$2{\cdot}8 \times 10^{-4}$	
3	300		$3{\cdot}7 \times 10^{-4}$	†

† Died.

a very rapid elimination of PNP from the plasma, the half-clearance being approximately 10 min. Attempts were then made to maintain the desired level in the plasma by injecting 100 mg PNP/kg subcutaneously, followed by a continuous subcutaneous administration of 180 mg/kg per hr with the aid of a motor-driven syringe, in rats anaesthetized with 35 mg sodium pentobarbitone per kg. The results re shown in Table 1; although an adequate concentration of PNP could be maintained in this way, the death of two out of three animals made us search for another method to reach our goal.

In a following experiment the possibility of obtaining a depot effect by using esters of PNP was investigated. PNP-acetate is very sparingly soluble, but PNP-butyrate is liquid at room temperature and may be injected as such. The plasma levels found after injecting 4·5 or 9 g of this ester per kg in one single subcutaneous injection are shown in Fig. 2, and Table 2 contains the concentrations of PNP found in the plasma, tumour, liver and brain of male Wistar rats with the mammary tumour injected with a single dose of 4·5–18 g of PNP-butyrate per kg. It is clear that a single injection of even 12 g does not guarantee the desired level of PNP in the tumour for more than 3 hr, and so a following series of rats were injected with multiple doses of PNP-butyrate either simultaneously at different sites or at different times. The results, which are

TABLE 2. THE *p*-NITROPHENOL (PNP) CONCENTRATION IN THE PLASMA, TUMOUR, LIVER, AND BRAIN OF WISTAR RATS WITH A MAMMARY TUMOUR AFTER ONE SUBCUTANEOUS INJECTION OF PNP BUTYRATE

Dose (g/kg)	Time after injection (hr)	Concentration (M × 10^4) of PNP in: plasma	tumour	liver	brain
4·5	1	4·4		5·2	1·4
	3	2·4		2·4	0
	3	1·9	1·9		
	5	2·6		1·5	
	24			0·7	0·7
6	3	2·2	2·2	2·8	
	3	2·2		1·9	
9	3	4·3		3·0	
	5	2·0	1·0		
	17	1·1	1·8		
	24	2·1	1·1		
	24	1·1	0·9		
12	1	2·4	1·7		
	3	2·5	3·0		
	3	2·3	0·8		
	3	1·9	1·3		
	3	2·3	2·9		
18*	5	3·3		0·9	

* This dose was very poorly tolerated.

summarized in Table 3, show that even these multiple injections were unable to provide the desired level of PNP in the tumour, although the amounts given were often toxic for the rat.

As the mammary tumour has a high lipid content it was hoped that compounds with a higher lipid solubility than DNP or PNP would reach higher concentrations in tumour tissue than these two compounds. Preliminary experiments with DNCa showed that the required concentration of this compound in the plasma could be obtained by injecting 15 mg/kg intravenously (see Fig. 2). However, even slightly higher doses of DNCa in a bicarbonate solution or substantially higher doses in arachis oil failed to provide the desired levels in the tumour (see Table 4).

The other uncoupling agent with a high lipid solubility which we studied, DNT, proved to be eliminated very slowly; a dose of 20 mg/kg (in 0·21 per cent aq. $NaHCO_3$

or in arachis oil) was well tolerated by adult rats, but when this dose was followed by two doses of 10 mg/kg (in arachis oil) 4 and 3 hr later, all three rats treated in this way died ½ hr after the last injection. It was concluded on the basis of these experiments that a dose of 25 mg/kg per 24 hr probably constituted the maximum dose which would be sustained well by our rats.

TABLE 3. THE *p*-NITROPHENOL (PNP) CONCENTRATIONS IN THE PLASMA, TUMOUR, LIVER AND BRAIN OF WISTAR RATS WITH A MAMMARY TUMOUR AFTER MULTIPLE INJECTIONS OF PNP BUTYRATE HAD BEEN GIVEN EITHER AT THE STATED INTERVALS OR SIMULTANEOUSLY AT DIFFERENT SITES

(Unless stated otherwise all injections were given subcutaneously.)

Mode of injection	Time (hr) after first injection	Concentration ($M \times 10^4$) of PNP in: plasma	tumour	liver	brain	Remarks
4·5 g/kg, divided over 3 sites						† 30 min after injection
4·5 g/kg, divided over 2 sites	3 3	3·4 2·3	 2·4	4·6	0·7	
1·5 g/kg, intraper. +3 g/kg subcut.	3	4·7		5·4	0·1	† 40 min after injection
4·5 g/kg, repeated after 12 hr	24	1·5	1·1			
9 g/kg, after 3hr 6 g/kg	5 5	2·8 2·3				
9 g/kg, repeated after 3 hr	5 5 5	3·7 4·2 5·7				Rat in poor condition
	—	—	—	—	—	† 4½ hr after 1st injection
	—	—	—	—	—	† 5 hr after 1st injection
12 g/kg, repeated after 3 hr	5 5	7·9 9·2				Both animals were in poor condition at time of killing

† Died.

A small series of normal rats and rats with the mammary tumour received daily subcutaneous injections of 10–25 mg of DNT in arachis oil (one rat received a dose of 30 mg) per kg, and the animals were killed at intervals of 1–24 hr after the last injection for the determination of the DNT concentration in the plasma and in the tumour. Two animals died spontaneously 1 and 2 hr after their last injection. The results, summarized in Table 5, indicate that even these relatively high doses were unable to provide a concentration in the tumour equalling or exceeding the level ($4·5 \times 10^{-4}$ M) required effectively to uncouple oxidative phosphorylation.

DISCUSSION

It is evident from these results that *in vitro* all four uncoupling agents studied are capable of increasing the rate of aerobic glycolysis in the Ehrlich ascites carcinoma to that of the anaerobic glycolysis, i.e. that they can abolish the Pasteur effect completely. However, our efforts to introduce the uncoupling agents in the tumour *in vivo* in the

TABLE 4. THE DINITROCARVACROL (DNCa) CONCENTRATION IN THE PLASMA AND TUMOUR OF WISTAR RATS WITH A MAMMARY TUMOUR AFTER ONE SUBCUTANEOUS (S.C.) OR INTRAPERITONEAL (I.P.) INJECTION

(DNCa was dissolved either in 0·21 per cent (w/v) aq.$NaHCO_3$, "Bic", or in arachis oil, "Oil".)

Dose (mg/kg) and route	Vehicle	Time after injection (hr)	Concentration ($M \times 10^4$) of DNCa: in plasma	in tumour	Remarks
30 i.p.	Bic	0·75	4·0		
30 s.c.	Bic	2	2·8		
30 s.c.	Bic	2	1·5	0·6	
30 i.p.	Bic	2	5·5		
100 s.c.	Oil	1	7·0		Four other rats thus treated died after 40 min (2 ×), 1 hr and 2 hr respectively
100 s.c.	Oil	3	4·2	1·8	
80 s.c.	Oil	4	4·2		Two other rats thus treated died after 14 and 24 hr, respectively
70 s.c.	Oil				† After 4 hr
60 s.c.	Oil	24	1·6		A third rat thus treated died after 1 hr
60 s.c.	Oil	1	3·9		
40 s.c.	Oil	0·5		1·4	Determined at autopsy, the rat died 30 min after the injection
40 s.c. repeated after 2 hr	Oil	4*	2·4	1·4	

* After first injection.
† Died.

TABLE 5. THE DINITROTHYMOL (DNT) CONCENTRATION IN THE PLASMA AND IN TUMOUR TISSUE AFTER ONE OR MORE SUBCUTANEOUS INJECTIONS OF A SOLUTION OF THIS COMPOUND IN ARACHIS OIL, IN NORMAL RATS AND WISTAR RATS WITH A MAMMARY TUMOUR

Animal	Dose (mg/kg) 1st day	2nd day	3rd day	4th day	5th day	Time after last injection (hr)	Concentration ($M \times 10^4$) of DNT: in plasma	in tumour
Rats with mammary tumour	25					24	1·12	0·42
	25	25				2†		1·80
	25	25	25			2	3·75	1·70
	25	25	25			1†		1·70
Normal rats	20	20	20			3	3·0	
	30					24	2·2	
	25	20	20	20		24	2·1	
	25	20	20	20	10	4	3·6	
	25	20	20	20	20	4	3·9	

† Died.

concentration required for this effect were not successful. In the case of DNP and PNP it was possible to realize this concentration in the plasma, but not in the tumour, without giving doses that were definitely toxic for the whole animal. The toxicities of DNCa and DNT were even greater so that the required concentration could not even be obtained in the plasma without endangering the life of the rat.

Uncoupling agents with a greater affinity for tumour tissue will therefore have to be found before it will be possible to test the hypothesis that such agents may arrest the growth of malignant cells *in vivo*, by depriving them of an important source of energy. In order to obtain an idea of the characteristics which such compounds should possess we compared some physical and biological characteristics of the four uncoupling agents used (see Table 6). This comparison brings out the fact that the activities of all

TABLE 6. COMPARISON OF SOME PHYSICAL AND BIOLOGICAL CHARACTERISTICS OF DINITROPHENOL (DNP), *p*-NITROPHENOL (PNP), DINITROCARVACROL (DNCa) AND DINITROTHYMOL (DNT)

Characteristic	DNP	PNP	DNCa	DNT
(1) p*K*	4·0	7·0	4·4	4·0
(2) Solubility in benzene at 20 °C (M)	0·45	0·12	1·87	3·73
(3) % bound to protein in ascites fluid	76	57	95	98
(4) Concentration ($M \times 10^4$) required to increase aerobic glycolysis to anaerobic level in				
(a) Ringer solution	0·5	1·5	0·3	0·05
(b) ascites fluid	3·6	4·7	4·8	4·5
(5) $\frac{(b)-(a)}{(b)} \times 100$	86	68	94	98
(6) Elimination from plasma: half clearance time (approx.)	2 hr	10 min	11 hr	13 hr
(7) Concentration required under 4(b) obtainable in plasma	yes	yes	no	no
(8) Concentration required under 4(b) obtainable in tumour	no	yes,* but poorly tolerated	no	no

* Provided PNP is injected as PNP-butyrate.

four compounds in ascites fluid *in vitro* are of the same order of magnitude, whereas their activities differ markedly when tested in the Krebs–Ringer–bicarbonate solution. The high activity exhibited by DNCa and DNT in the latter medium led us to expect a high activity in ascites fluid too, but here their activity is probably reduced by a high affinity to protein. On the other hand, the activity of these two compounds may be increased by their relatively high lipid solubility, favouring absorption by the tumour cells. Assuming that in ascites fluid only that fraction of the uncoupling agent is active, which is not protein bound, the concentration of the inactive fraction in ascites fluid can be found by subtracting the concentration uncoupling oxidative phosphorylation in Krebs–Ringer–bicarbonate solution from the corresponding concentration in ascites fluid. This difference expressed as a percentage of the concentration required in ascites fluid agrees reasonably well with the percentage bound to protein as determined more directly, viz. by ultrafiltration of ascites fluid to which the uncoupling compound had been added.

It is interesting to note the negative correlation between the affinity to protein and the speed of elimination from the plasma expressed as the half clearance.

On the basis of these admittedly limited observations we should like to draw the tentative conclusion that the uncoupling agents to be applied parenterally against malignant cells should preferably have a minimum affinity for protein, and need not have a higher lipid solubility than PNP in order to reach the tumour from the blood stream in sufficient quantity. The rapid elimination from the plasma exhibited by the latter compound may be due partly to diffusion into the extracellular tissue fluid or into the cells, and as such may be termed desirable. In so far as it is accounted for by elimination in the urine it is of course a disadvantage, which may be met by the use of esters or other kinds of depot preparation, as exemplified by the results obtained with PNP-butyrate.

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SHORT COMMUNICATIONS

The influence of epinephrine and norepinephrine on the accumulation of amphetamine-1-^{14}C by rat brain*

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IN THE course of recent experiments in this laboratory on the distribution of amphetamine in brain,[1] it was found that this substance was rapidly accumulated by brain and rapidly removed. Amphetamine therefore appeared to be a good indicator for studies on the effect of various treatments on the process of blood-brain transport. This report describes the positive effect of large doses of epinephrine and norepinephrine on the accumulation of amphetamine by brain.

A solution consisting of dl-amphetamine-1-^{14}C (0·3–0·7 μc/ml) and unlabeled dl-amphetamine to give a final concentration of free base of 0·9 μmole/ml was prepared in 0·9 per cent saline.† Other compounds tested were dissolved in this solution, all at a concentration of 0·75 μmole/ml. These large concentrations were employed since catecholamines are so rapidly metabolized.[2, 3] Fasted Wistar male rats (150–250 g) were injected intraperitoneally with 1 ml of the appropriate solutions per 100 g of body weight. At $\frac{1}{2}$ hr after injection, when the maximal amount of ^{14}C appeared in brain following the injection of amphetamine alone, the animals were decapitated and blood was collected in glassware dusted with anticoagulant. Brain tissue anterior to the first spinal nerve was removed, washed in saline, blotted and weighed. Ten per cent homogenates were prepared in 0·1 N HCl containing 4μg of amphetamine/ml to act as carrier. Brain-^{14}C was recovered and identified as amphetamine in the following manner. Protein was precipitated with trichloroacetic acid, and the supernatant solution obtained was extracted with benzene at pH 12–13.[4] The amine was recovered from the benzene by extraction with 0·1 N HCl. After freeze-drying, the residue was spotted on Whatman No. 1 paper and developed (descending technique) in *n*-butanol : acetic acid : water (50 : 40 : 10). Radioactivity of dried samples was combusted to CO_2 and measured as ion current with a vibrating reed electrometer (1 μc = $4{\cdot}62 \times 10^{-12}$ amperes).

The main findings are shown in Table 1. Both epinephrine and norepinephrine, but not normetanephrine, the major metabolite of norepinephrine,[5] definitely increased the concentration of amphetamine in brain. These are noteworthy effects in view of the difficulty in modifying the transport of compounds into brain.[5] The good recoveries of ^{14}C, which partitioned like amphetamine according to established procedures,[4] and the detection of single peaks of radioactivity corresponding to authentic amphetamine, make it very probable that the ^{14}C accumulated was amphetamine. Plasma radioactivity from a few animals was determined in order to evaluate the contribution of blood-^{14}C to the brain values. Assuming a 2·4 per cent contamination,[6] less than 1 per cent of the total brain-^{14}C could be attributed to blood. Direct determination of blood in brain[7] gave the following values: amphetamine-treated, 0·8 per cent; amphetamine + epinephrine, 0·8 per cent; amphetamine + norepinephrine, 0·9 per cent. The data on radioactivity of plasma, to date, however, do not permit an evaluation of the role of circulating ^{14}C in the production of increased content of amphetamine in brain by catecholamines.

The animals injected with amphetamine + epinephrine, or with amphetamine + norepinephrine, were unresponsive to external stimuli and somewhat ataxic, these effects being more pronounced in the animals treated with norepinephrine. This behavior contrasts with the hyperexcitability of animals injected with the same dose of amphetamine or amphetamine + normetanephrine. Control animals injected with epinephrine or norepinephrine alone were quiescent relative to animals injected with saline, but they were more alert than those given both amphetamine and catecholamine. This diminished alertness in the latter animals, however, could not have been due to the increase of

* This investigation was supported in part by the National Institutes of Health (B-1895), and in part by the Association for the Aid of Crippled Children.

† The amphetamines were kindly supplied by Smith, Kline and French Laboratories.

amphetamine in brain, since animals injected with amphetamine in doses twice those previously used, i.e., 1·8 μmoles/ml, had higher levels of amphetamine in brain than did those described in the table, and exhibited typical excitement. The possibilities that amphetamine promoted an increase in the concentrations of catecholamines in the brain, or that increased brain catecholamines resulted directly from the large doses employed, were not investigated.

Despite the lack of accumulation of epinephrine in brain during intravenous infusion, except to a very limited degree in the hypothalamus,[6] it is possible that the observed behavior of the rats is due to elevated cerebral catecholamines. This possibility must be considered for the following reasons:

(1) it has been reported that when epinephrine or norepinephrine is injected into the lateral ventricle anesthetic-like effects are produced;[5]

(2) it may be that the behavior change is mediated by changed hypothalamic concentrations of the catecholamines;[6]

(3) since amphetamine has a profound influence on the metabolism of the catecholamines,[3] general or local changes in the brain concentration of either epinephrine or norepinephrine, or both, may occur when amphetamine is administered together with the catecholamines.

TABLE 1. INFLUENCE OF EPINEPHRINE AND NOREPINEPHRINE ON THE ACCUMULATION OF AMPHETAMINE-1-^{14}C BY RAT BRAIN

Substance administered	Accumulation of ^{14}C by rat brain*	Percent recovery brain-^{14}C as amphetamine	R_f brain-^{14}C†
Amphetamine	1·07 ± 0·03 (8)	94	0·70–0·81 max = 0·72–0·74
Amphetamine + norepinephrine	1·49 ± 0·07 (8) $P < 0·005$	90	0·71–0·80 max = 0·73–0·76
Amphetamine + epinephrine	1·57 ± 0·09 (3) $P < 0·05$	90	0·69–0·80 max = 0·73–0·76
Amphetamine + normetanephrine	0·82, 0·86	—	—

* $\left(\frac{\text{Total brain-}^{14}\text{C/g wet wt}}{^{14}\text{C administered/kg body wt}} \times 10^3\right)$; mean values from number of animals in parentheses ± S.E.; *P*-values for comparisons with amphetamine alone.

† R_f of authentic amphetamine-^{14}C, 0·66–0·81; max = 0·76.

The effects shown suggest that epinephrine and norepinephrine, or perhaps metabolic intermediates, might regulate the uptake of at least some compounds by brain. That these effects might be more general is implied by the findings of others,[10] and is being investigated further with other classes of compounds. In the event that similar findings result from the continuous infusion of physiological quantities of catecholamines or by other interventions that may maintain a relatively high level of these compounds in blood, the data suggest the interesting possibility that the degree of action of amphetamine (and possibly of other neuro-active agents) might be regulated by circulating catecholamines.

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Inhibition of oxidative phosphorylation by an antipyretic drug

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IN THE course of a study on the mechanism of the pharmacological action of antipyretics, an inhibitory effect "in vivo" on rat oxygen uptake and "in vitro" on isolated rat liver mitochondria was observed using 2-allyl-oxybenzamide, a salicylamide (2-hydroxybenzamide) derivative.[1]

The main results, obtained with rat liver mitochondrial preparations, are summarized in this report. Table 1 shows the effect of 2-allyl-oxybenzamide (AOB)* on the oxidative phosphorylation

TABLE 1. OXIDATIVE PHOSPHORYLATION IN PRESENCE AND IN ABSENCE OF 2-ALLYLOXYBENZAMIDE (AOB)

	substrate	AOB	O_2 uptake (μatoms/mg N)	P esterified (μmoles/mg N)	P/O
(a)					
	α-ketoglutarate	—	12·59	31·44	2·50
	α-ketoglutarate	0·002 M	3·90	10·51	2·70
	β-hydroxybutyrate	—	4·70	13·20	2·80
	β-hydroxybutyrate	0·002 M	1·67	4·30	2·57
	succinate	—	16·30	29·80	1·83
	succinate	0·002 M	16·50	23·60	1·43
(b)					
	DPNH	—	10·10	10·67	1·06
	DPNH	0·002 M	9·10	8·07	0·89
(c)					
	ferrocytochrome C	—	5·76	2·70	0·47
	ferrocytochrome C	0·002 M	6·10	2·74	0·45

The oxygen uptake was measured manometrically at 26 °C in the Warburg apparatus. The vessels contained 0·005 M $MgSO_4$, 0·03 M glucose, 0·0014 M ATP, 0·8 mg yeast hexokinase (Sigma, type II), 0·00001 M cytochrome C, 0·09 M sucrose, and:

(a) α-ketoglutarate (0·01 M), succinate (0·01 M) and β-hydroxybutyrate (0·02 M) as substrates, 0·03 M potassium phosphate buffer, pH 7.4, 0·00036 M $MnCl_2$, mitochondria (isolated in 0·25 M sucrose) 1 mg of N; time of incubation 20 min.;

(b) DPNH (0·002 M) as substrate, 0·009 M potassium phosphate buffer, pH 7·4, 0·04 M *tris* buffer, pH 7·4, 0·001 M ethylendiaminotetracetate, mitochondria (isolated in 0·25 M sucrose and pretreated for 15 min. at 0 °C with 0·075 M sucrose) 0·5 mg of N; time of incubation 15 min.;

(c) reduced cytochrome C as substrate, 0·009 M potassium phosphate buffer, pH 7·4, 0·02 M *tris* buffer, pH 7·4, 0·01 M ascorbate, 0·00036 M $MnCl_2$, 0·01 M KF, mitochondria (isolated in 0·25 M sucrose and pretreated for 15 min. at 0 °C with 0·075 M sucrose) 1 mg of N; time of incubation 30 min.;

* 2-allyl-oxybenzamide was kindly supplied by Cassella-Curta (Frankfurt/M).

final volume 2·8 ml; gas phase air; KOH and filter paper in the centre well.

supported by several substrates, included added reduced diphosphopyridine nucleotide (DPNH) and ferrocytochrome C. It is evident that AOB (0·002 M) produces a marked inhibition of respiration with α-ketoglutarate and β-hydroxybutyrate as substrates; the same concentration of AOB is without effect on the oxidation of succinate, added DPNH and cytochrome C reduced by ascorbate.

The possibility of two alternative pathways for oxidation of added DPNH may explain the failure of AOB to produce inhibition on oxygen uptake with this substrate, but the partial depression of the coupled phosphorylation, observed in hypotonically-treated mitochondria, may indicate that the internal pathway is AOB-sensitive; in this case the phosphorylation observed in the presence of AOB could originate in a great degree from the oxidation of cytochrome C, reduced by *external* pathway (P/O = 0·89).

With α-ketoglutarate and β-hydroxybutyrate as substrates, AOB inhibits electron transport, without affecting the efficiency of the coupled phosphorylation, while with succinate the P/O ratio is slightly reduced (this effect might be due to inhibition of oxidation of fumarate derived from succinate). AOB was found to have no uncoupling effect on the phosphorylation coupled to the ferrocytochrome C oxidation if the P/O ratios are corrected for endogenous phosphorus uptake; the endogenous phosphorus uptake, observed in the absence of ascorbate and cytochrome C, is partially inhibited by AOB.

The action of AOB is also present in a ~ P acceptors-deficient system activated by 2, 4 dinitrophenol and in water pre-incubated mitochondria oxidizing β-hydroxybutyrate in the presence of diphosphopyridine nucleotide (DPN) but in the absence of cytochrome C. In this case (water pre-incubated mitochondria) the addition of cytochrome C partially removes the effect of AOB; the antagonism is more evident if at the same time the level of added DPN is increased. Data obtained with these experiments are plotted in Fig. 1.

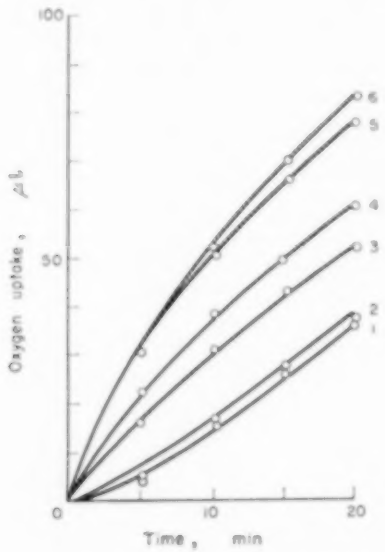

FIG. 1. The effect of AOB on oxygen uptake of water-pretreated mitochondria. The vessels contained 0·03 M potassium phosphate buffer, pH 7·4, 0·005 M $MgSO_4$, 0·001 M ethylendiaminotetracetate, 0·02 M β-hydroxybutyrate, mitochondria (isolated in 0·25 M sucrose and pre-incubated in water for 30 min. at 0 °C) 1 mg of N; other conditions as indicated in Table 1. Additions: (1) 0·002 M AOB + 0·00036 M DPN; (2) 0·002 M AOB + 0·0014 M DPN; (3) 0·002 M AOB + 0·00036 M DPN + 0·00002 M cytochrome C; (4) 0·002 M AOB + 0·0014 M DPN + 0·00002 M cytochrome C; (5) 0·00036 M DPN or 0·00036 M DPN + 0·00002 M cytochrome C; (6) 0·0014 M DPN or 0·0014 M DPN + 0·00002 M cytochrome C.

It may also be pointed out that cytochrome C is unable to remove the inhibition of respiration in phosphorylating mitochondria and that in water pre-treated mitochondria oxidizing succinate the drug is ineffective both in the presence and in the absence of cytochrome C.

The reversal of AOB inhibition by added cytochrome C may be explained if one considers cytochrome C as an electron acceptor bypassing the point of attachment of the inhibitor (AOB) to the electron transport chain. With water pre-treated mitochondria cytochrome C may be thought to accept electrons directly from flavoproteins as it has been observed in disrupted mitochondria.[2, 3] In these conditions, with amytal (5-ethyl-5-isoamylbarbituric acid) are obtained similar results.

The present data appear to support the hypothesis that AOB inhibits electron transport in the respiratory chain, without affecting the coupled phosphorylation reactions and suggest that the effect of the drug on mitochondrial respiration might be due to the inhibition of DPNH-cytochrome C reductase. In this respect, AOB has an action similar to that of amytal.[4]

In regard to the pharmacological implications, these experiments can support the following considerations:

(a) the inhibition of respiration by AOB might play an important part in the antipyretic effect of this substance in as much as it might lead to reduced heat formation;

(b) the allyl group is important for the inhibition of oxygen uptake. In this respect salicylamide (2-hydroxybenzamide) is much less active and this substance differs from AOB in that a OH replaces the allyl group. It is of interest to note that AOB is an antipyretic more active than salicylamide;

(c) since AOB has a strong depressing action upon central nervous system, the present data confirm the probability that the inhibition of DPNH-cytochrome C reductase can be related to the depression of central nervous system induced by several drug (hypnotics, sedatives, tranquillizers).

Indeed, the action of AOB is similar to that of amytal and, in some way, it resembles the action of chlorpromazine.[5]

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BOOK REVIEW

Inhibition in the Nervous System and Gamma-aminobutyric Acid (E. ROBERTS: Editor) Pergamon Press, Ltd., Oxford, 1960, 600 pp, 100s.

THIS important book will be read with profit by many who take an interest in biochemical pharmacology. The main topics discussed are at the point of intersection of biochemistry, physiology and pharmacology. Over twenty years ago Harriette Chick and her colleagues first described the characteristic epileptiform convulsions of vitamin B6-deficient animals. The role of pyridoxine in the metabolism of glutamic acid is now well established, and much support is given by some contributors to this Symposium to the idea that a failure of the enzyme responsible for the conversion of L-glutamic acid to γ-aminobutyric acid (GABA) is at the bottom of this neurological disorder, although there is by no means general agreement on this.

It is not easy to select a general theme to cover this collection of over seventy individual contributions, but we may come nearest when we quote the concluding sentences of K. A. C. Elliott's paper: "I do believe that we must now recognise the fact that substances which are concerned in the conditioning of nervous excitability can be intermediary metabolites. The fields of intermediary and energy metabolism and of functional activity cannot longer be separated in our studies of the nervous system."

The theory of chemical transmission of nervous impulses is derived from the experimental analysis of the activity of the peripheral effector nerves. We have at present no satisfactory evidence based on the study of the central nervous system. And yet, so firmly is this theory fixed in contemporary thought that it appears essential also when the functioning of central synapses is discussed. Reading this book makes us understand why the idea of specific and localised interaction between "transmitter" and "receptor" is such an indispensable concept. However, much of the material indicates that we have also to reckon with interactions between naturally occurring substances (or drugs) and neuronal membranes not confined to the synapses and which either raise or lower the excitability of the neurone. Is this kind of interaction also restricted to certain types of neurone, and how does it fit in with the picture of specific inhibitor pathways? These are some of the questions raised, but by no means definitely answered.

An important gain that we owe to some of the members of this Symposium is the discovery of the metabolic pathway that involves formation and breakdown of GABA and the more recent finding that in nervous tissue it represents a quantitatively significant alternative to the classical pathway of succinate formation in the tricarboxylic acid cycle. In spite of all the evidence that shows that the intermediates of the "GABA shunt" are partly held in separate intracellular compartments, it seems unlikely that a major metabolite like GABA acts as a true transmitter, but there are many observations, on invertebrate as well as on vertebrate preparations, that point to the presence of compounds related to GABA that may have transmitter functions in inhibitory neurones. The discovery of such a compound would be an important contribution to the problem of inhibition in the nervous system; the complexity of this problem is well illustrated by many contributions to this volume.

H. BLASCHKO

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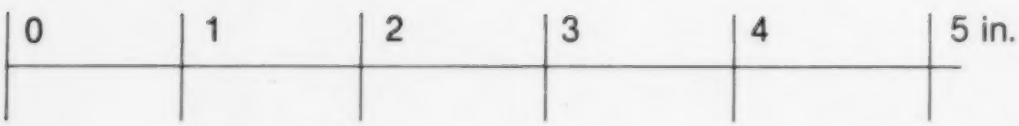

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